UNIVERSIDAD AUTÓNOMA DE MADRID
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Envejecimiento prematuro en pacientes VIH+: evaluación *in vitro* e *in vivo* del impacto del tratamiento con análogos de nucleós(t)idos en la longitud de los telómeros

Tesis Doctoral

Natalia Carolina Stella Ascariz

Madrid, 2018

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Memoria presentada para optar al grado de Doctor por

Natalia Carolina Stella Ascariz

Licenciada en Biología

Directores:

Dr. José Ramón Arribas

Dr. Jesús Mingorance

Agradecimientos

Muchas personas han formado parte de esta tesis de manera directa o indirecta y a todos ellos les quiero dar mi más sincero agradecimiento.

En primer lugar a mis directores de tesis, Mingo y José Ramón, por darme esta oportunidad, guiarme a lo largo de este aprendizaje y enseñarme a no bajar los brazos.

A Julio García y todo el Servicio de Microbiología del Hospital Universitario La Paz por acogerme en el servicio durante todos estos años.

A todo el personal de la Consulta de Medicina Interna II que me han ayudado en el trabajo día a día, sin ellos la recogida de muestras no hubiera sido posible.

A Francisco Gaya quién me ha facilitado mucho el trabajo gracias al diseño de su base de datos.

A Rosario Perona por permitirme realizar parte de este trabajo en su laboratorio y a todos mis compañeros del IIB, especialmente a Laura por trabajar conmigo hasta cualquier hora para tener los resultados a tiempo.

A todos mis compañeros del laboratorio de Microbiología y del IdiPAZ. Un especial agradecimiento a Natalia, Elena y Alma que aunque ya no están me han acompañado en gran parte de este camino.

A todo el grupo de investigación de SIDA y enfermedades infecciosas del IdiPAZ. Esta tesis ha sido posible gracias al trabajo de todos ellos. Un especial agradecimiento a Rocío, que además de ser una gran compañera es una gran amiga.

A María Jesús García por su incondicional ayuda cuando terminé la carrera y por permitirme dar mi primer paso en la investigación a su lado.

Finalmente quiero agradecer a mi familia por apoyarme y creer en mí siempre.

Resumen

Gracias al indiscutible éxito del tratamiento antirretroviral (TAR) la esperanza de vida del paciente VIH ha aumentado significativamente hasta aproximarse a la de la persona no infectada. Sin embargo, el TAR no consigue restaurar por completo la salud del paciente por lo que la población VIH+ presenta múltiples comorbilidades asociadas al envejecimiento a edades menos avanzadas que en la población no VIH. Dado que en población general existe una asociación entre las enfermedades asociadas al envejecimiento y el acortamiento telomérico, y además existe una similitud estructural y funcional entre la transcriptasa inversa del VIH y la telomerasa celular, el trabajo realizado en esta tesis doctoral se ha centrado en el estudio del impacto de los inhibidores de la transcriptasa inversa análogos de nucleós(t)idos (N(t)RTIs) en la actividad telomerasa *in vitro* y su impacto en la longitud telomérica (LT) *in vivo* tanto en pacientes con prolongada supresión virológica como pacientes naïve que comienzan tratamiento antirretroviral (TAR) de primera línea.

En el estudio *in vitro* hemos confirmado que concentraciones terapéuticas de los N(t)RTIs tenofovir (TFV), principio activo del fármaco antirretroviral tenofovir difumarato (TDF), y abacavir (ABC) pero no de emtricitabina (FTC) producen una disminución de la actividad telomerasa dosis-dependiente, siendo TFV el inhibidor más potente. Esta inhibición no se asocia a cambios en los niveles de proteína ni de la expresión génica de los componentes de la telomerasa o del complejo shelterina.

En los estudios *in vivo*, realizados tanto en la cohorte de pacientes con supresión virológica prolongada como en los pacientes naïve que comienzan TAR de primera línea, se observó de forma global una ganancia de la LT en sangre tras dos años de seguimiento. Al analizar la LT en función del régimen del TAR, en el caso de los pacientes VIH+ con prolongada supresión viral la LT en sangre aumentó menos en el grupo de pacientes expuestos a TDF o ABC que en el grupo tratado con la pauta ahorradora de N(t)RTIs. Por el contrario, en los pacientes naïve la LT en sangre aumentó más en el grupo de pacientes tratados con TDF/TFC que en el grupo tratado con la pauta ahorradora de N(t)RTIs. Estas diferencias sugieren que los N(t)RTIs producen una mejor recuperación inicial de la inmunosenescencia asociada a la infección por el VIH; sin embargo interfieren con la recuperación a largo plazo, posiblemente debido a su capacidad para inhibir la telomerasa.

Abstract

Due to the success of antiretroviral treatment (ART), the life expectancy of the HIV patient has increased significantly, being close to the uninfected person. However, ART does not fully restore the health of patient, so the HIV population has multiple comorbidities associated with aging at less advanced ages than the non-HIV population. Given that in the general population there is an association between diseases associated with aging and telomeric shortening, and there is also a structural and functional similarity between HIV reverse transcriptase and cellular telomerase, the work carried out in this doctoral thesis has focused on the study of the impact of nucleoside and nucleotide reverse transcriptase inhibitors (N(t)RTIs) on telomerase activity in vitro and its impact on telomere length (TL) in vivo in patients with prolonged virological suppression as well as naïve patients who start first-line antiretroviral (ART) treatment.

In the in vitro study we have confirmed that therapeutic concentrations of the N(t)RTIs such as tenofovir (TFV), the active substance of the antiretroviral drug tenofovir difumarate (TDF), and abacavir (ABC) but not of emtricitabine (FTC) produce a decrease in dose-dependent telomerase activity, TFV being the most potent inhibitor. This inhibition is not associated with changes in protein levels or gene expression of the components of telomerase or shelterin complex.

In in vivo studies, carried out both in the cohort of patients with prolonged virological suppression and in naïve patients who started first-line ART, a gain in TL in blood was observed overall after two years of follow-up. When TL was analyzed according to the ART regimen, in the case of HIV patients with prolonged viral suppression, blood TL increased less in the group of patients exposed to TDF or ABC than in the group treated with the N(t)RTIs-sparing regimen. Conversely, in naïve patients the TL in blood increased more in the group of patients treated with TDF/TFC than in the group treated with the N(t)RTIs-sparing regimen. These differences suggest that N(t)RTIs produce a better initial recovery of immunosenescence associated with HIV infection; however, they interfere with long-term recovery, possibly due to their ability to inhibit telomerase.

Índice

Abreviaturas

3TC	Lamivudina
A	Adenina
ABC	Abacavir
ADN	Ácido desoxirribonucleico
ARN	Ácido ribonucleico
AZT	Zidovudina
C	Citidina
CV	Carga viral
d4T	Estavudina
ddI	Didanosina
DDR	Respuesta al daño del ADN (*DNA damage response*)
DRV	Darunavir
DRV/r	Darunavir/ritonavir
FTC	Emtricitabina
G	Guanosina
LT	Longitud telomérica
N(t)RTIs	Inhibidores de la transcriptasa inversa análogos de nucleós(t)idos (*Nucleoside and nucleotide reverse transcriptase inhibitors*)
NNRTIs	Inhibidores de la transcritasa inversa no análogos de nucleósidos (*Non-nucleoside reverse-transcriptase inhibitors*)
PBMCs	Células mononucleares de sangre periférica (*Peripheral blood mononuclear cells*)
POT1	Proteína de protección del telómero 1
RAL	Raltegravir
RAP1	Proteína activadora represora 1
T	Timidina
TAR	Tratamiento antirretroviral
TDF	Tenofovir difumarato

TERC	Componente de ARN de la telomerasa (*Telomerase RNA component*)
TERT	Transcriptasa inversa de la telomerasa (*Telomerase Reverse Transcriptase*)
TFV	Tenofovir
TIN2	Proteína nuclear 2 de interacción con TRF1
TPP1	Proteína de interacción con POT1
TRF1	Proteína de unión a repeticiones teloméricas 1
TRF2	Proteínas de unión a repeticiones teloméricas 2
VIH	Virus de la inmunodeficiencia humana.

Introducción

El envejecimiento es un proceso natural, y desde una perspectiva biológica se define como el deterioro progresivo de la función fisiológica que se produce como consecuencia del daño acumulado tanto a nivel molecular como celular, repercutiendo finalmente en el deterioro de la función a nivel de tejidos y órganos. Este proceso invariablemente resulta en una disminución de las respuestas al estrés, una mayor susceptibilidad a las enfermedades, un incremento de la fragilidad y finalmente la muerte. Las manifestaciones clínicas incluyen pérdida en la función orgánica (hígado, riñón, corazón, etc), pérdida ósea (osteoporosis), pérdida de masa muscular (sarcopenia), disminución neurocognitiva (demencia) y pérdida de la función inmunológica (inmunosenescencia). Este deterioro progresivo es el principal factor de riesgo de diversas patologías, incluido el cáncer, la diabetes, los trastornos cardiovasculares y las enfermedades neurodegenerativas.

Los mecanismos moleculares y celulares, que contribuyen al proceso de envejecimiento y al desarrollo de las enfermedades crónicas asociadas a la edad, son la acumulación de alteraciones y daño en el ácido desoxirribonucleico (ADN), el acortamiento telomérico, las alteraciones epigenéticas, la pérdida de proteostasis, la desregulación de los mecanismos de detección de nutrientes, la disfunción mitocondrial, la senescencia celular, la disminución de la capacidad regenerativa de los tejidos y la inflamación [1] (Figura 1). Estos mecanismos se agrupan en tres categorías: mecanismos primarios, antagónicos e integradores. Los mecanismos primarios, que incluyen la inestabilidad genómica, el acortamiento telomérico, alteraciones

Figura 1 | Mecanismos moleculares y celulares del envejecimiento. Mecanismos primarios: inestabilidad genómica, acortamiento telomérico, alteraciones epigenéticas y pérdida de proteostasis. Mecanismos antagónicos: desregulación de los mecanismos de detección de nutrientes, disfunción mitocondrial y senescencia celular. Mecanismos integradores: disminución de la capacidad regenerativa de tejidos e inflamación. Figura adaptada de [1].

epigenéticas y la pérdida de proteostasis, se caracterizan por el hecho de ser inequívocamente negativos ya que son las principales causas del daño celular. Por el contrario, los mecanismos antagónicos son aquellos que desencadenan respuestas compensatorias o antagónicas al daño. Estas respuestas inicialmente mitigan el daño, pero si son exacerbadas o se cronifican, finalmente se vuelven dañinas. Dichos mecanismos son la desregulación de los mecanismos de detección de nutrientes, la disfunción mitocondrial y la senescencia. Por último, los mecanismos integradores, compuestos por la disminución de la capacidad regenerativa de tejidos y la inflamación, son el resultado final de los mecanismos previos y en última instancia los responsables del deterioro funcional asociado al envejecimiento.

Envejecimiento prematuro del paciente VIH+

El desarrollo de la terapia antirretroviral para el tratamiento de la infección por el virus de la inmunodeficiencia humana (VIH) ha sido uno de los mayores logros de la medicina moderna. El éxito del tratamiento antirretroviral (TAR) en la mejora de la función inmune y de la prevención de la morbimortalidad asociadas a eventos SIDA ha supuesto un aumento de la esperanza de vida del paciente VIH+ de unos pocos años a décadas, lo que ha cambiado radicalmente el pronóstico de la enfermedad de mortal a crónica. UNAIDS ha estimado que en todo el mundo hay 3,6 [3,2-3,9] millones de personas de ≥50 años de edad con infección por el VIH y que esta población se ha ido incrementando en los últimos años [2]. Al considerar solamente los países con altos ingresos (Europa central y occidental, Estados Unidos y Canadá) el 33 % de la población VIH+ tiene 50 años o más [2] y en España concretamente se ha estimado que el 46,5% de la población VIH+ corresponde a dicho estrato de edad [3]. Sin embargo, a pesar del indiscutible éxito del TAR, éste no consigue restaurar por completo la salud del paciente. Incluso en aquellos con adecuada respuesta al tratamiento, se produce un aumento del riesgo de comorbilidades no asociadas a la infección por VIH sino relacionadas con la edad y con la inflamación crónica tales como cáncer, enfermedad cardiovascular, enfermedad renal, enfermedad hepática, enfermedad ósea y deterioro neurocognitivo [4]. No se sabe si el mayor riesgo de padecer comorbilidades no asociadas a la infección por VIH es una expresión de un envejecimiento acelerado, las comorbilidades ocurren a edades más tempranas, o si por el contrario se debe a un envejecimiento acentuado debido a una mayor prevalencia de dichas comorbilidades en cada estrato de edad [5]. En un estudio de casos y controles, Guaraldi *et al* han determinado que la prevalencia de múltiples comorbilidades en la población VIH+ es equivalente a la observada en la población general con una edad de 10-15 años más avanzada [6]. Por otro lado, dos estudios recientes han establecido, mediante biomarcadores epigenéticos del envejecimiento, que la

población de pacientes con infección por VIH presenta un fenotipo equivalente al de una población no VIH cinco años mayor [7,8], por lo que la idea de envejecimiento prematuro ha ganado peso en los últimos años.

Se han propuesto varios factores que podrían contribuir al mayor riesgo de presentar comorbilidades no asociadas al VIH, incluyendo los factores de riesgo tradicionales (uso de tabaco, alcohol o drogas) que son más prevalentes en la población VIH+, la toxicidad del TAR y la activación inmune e inflamación persistentes que se detectan incluso en los pacientes en TAR con supresión virológica prolongada [9].

Otro factor potencialmente asociado al envejecimiento prematuro del paciente VIH+ es el acortamiento telomérico. En población general se ha observado una estrecha asociación entre el acortamiento telomérico en sangre y enfermedades propias del envejecimiento, como son la enfermedad cardiovascular, la demencia y el cáncer [10–12]. Además, en múltiples estudios se ha observado que la población VIH+ presenta telómeros más cortos que los controles no VIH [13–16]. El mayor acortamiento telomérico en pacientes VIH+ puede deberse a la inhibición de la actividad de la telomerasa humana, enzima responsable del mantenimiento de la longitud de los telómeros, por los fármacos antirretrovirales; concretamente por los inhibidores de la transcriptasa inversa análogos de nucleós(t)idos (N(t)RTIs).

Longitud telomérica en patologías asociadas al envejecimiento

Diversos estudios han mostrado que existe una relación inversa entre la longitud telomérica (LT) y la edad de un individuo [17–20]. Asimismo, se ha observado que la LT es mayor en mujeres que en hombres [19,21,22]. Aunque la evidencia es inconsistente sobre el grado de heredabilidad, se considera que la LT es heredable [23,24], y modificable por factores del entorno y del estilo de vida [25]. Se ha determinado que factores tales como la obesidad [26], el tabaquismo [27], el consumo de alcohol [28], la terapia de reemplazo hormonal [29] y la existencia de una enfermedad inflamatoria crónica [21] se asocian a una menor LT como consecuencia del aumento de los niveles de estrés oxidativo e inflamación [26,30].

Estudios epidemiológicos han determinado que la presencia de una menor LT determinada en sangre total se asocia con múltiples causas de mortalidad, así como también con patologías asociadas al envejecimiento tales como la enfermedad cardiovascular, algunos cánceres, la obesidad, el Alzheimer, la diabetes mellitus, la demencia y la osteoporosis [10,11,31–35]. Además, existen ciertas patologías humanas, definidas como telomeropatías o síndromes teloméricos, que muestran un acortamiento telomérico prematuro como consecuencia de

mutaciones en los genes que codifican los factores implicados en el mantenimiento de los telómeros. Las telomeropatías humanas se asocian principalmente con el síndrome de Hoyeraal-Hreidarsson, disqueratosis congénita, anemia aplásica, fibrosis pulmonar idiopática y fibrosis hepática. Aunque estas enfermedades muestran una amplia y compleja variedad de síntomas clínicos, todas ellas se caracterizan por presentar telómeros críticamente cortos. La edad de inicio y la gravedad de las manifestaciones clínicas varían entre los individuos. Estos síndromes se caracterizan por la pérdida prematura de la capacidad regenerativa de los tejidos, afectando a los tejidos con tasas de proliferación altas y bajas [36,37].

Telómero

Los extremos de los cromosomas eucariotas están formados por una estructura especial de heterocromatina, conocida como telómero, que los protege de la degradación y recombinación cromosómica. Por lo tanto, el telómero es una estructura esencial para la estabilidad del cromosoma. En la especie humana, está constituido por repeticiones en tándem de una secuencia rica en guaninas (TTAGGG). El número de repeticiones de dicha secuencia es variable y su longitud en el momento del nacimiento es alrededor de 10-15kb por telómero [12]. Además, su estructura se caracteriza por la presencia de un extremo protuberante de 150-200 nucleótidos en el extremo 3’ de la cadena rica en guaninas [12,38,39] (Figura 2a). Este extremo monocatenario invade la región bicatenaria del telómero, generando una estructura en bucle conocida como el bucle-t, que oculta el extremo 3' monocatenario del telómero evitando que se active la respuesta al daño del ADN (DDR, de sus siglas en inglés *DNA damage response*) [40] (Figura 2b). Asociado a las repeticiones teloméricas, se encuentra un complejo multiproteico conocido como “shelterina” que tiene un papel fundamental en la estabilización y protección de los telómeros y en la regulación de su longitud. El complejo shelterina está formado por seis proteínas: las proteínas de unión a repeticiones teloméricas 1 (TRF1) y 2 (TRF2) que se unen al ADN bicatenario, la proteína de protección del telómero 1 (POT 1) que reconoce los extremos monocatenarios protuberantes, la proteína nuclear 2 de interacción con TRF1 (TIN2) que interacciona con TRF1 y TRF2 y estabiliza la asociación entre ambas, la proteína de interacción con POT1 (TPP1) que conecta POT1 con TIN2 y la proteína activadora represora 1 (RAP1) que interacciona con TRF2 y favorece la unión de TRF2 al ADN telomérico [41–44] (Figura 2c). TRF1 tiene una importante función en el control la replicación de los telómeros, TRF2 es requerida para la formación y estabilidad del bucle-t y POT 1 evita que el telómero sea reconocido como ADN dañado y la consecuente activación de la DDR.

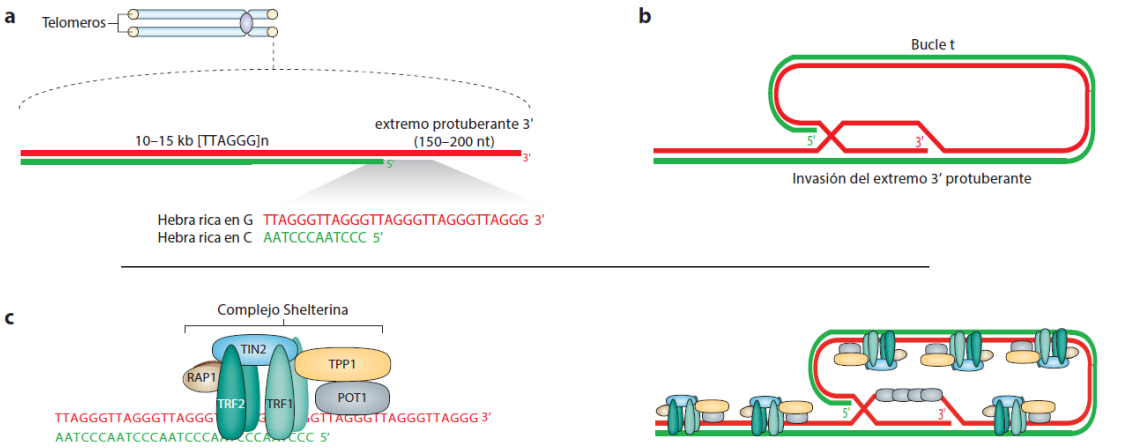

Figura 2 | Estructura del telómero. (a) En humanos los telómeros están formados por repeticiones en tándem de la secuencia TTAGGG. La hebra rica en guaninas presenta un extremo 3' monocatenario protuberante de aproximadamente 150-200 nucleótidos. (b) Esquema de la estructura de bucle-t. El bucle-t se forma tras la invasión del extremo protuberante 3' en el ADN telomérico bicatenario. (c) El complejo shelterina está formado por seis proteínas. TRF1 y TRF2 se unen a las repeticiones teloméricas de doble cadena, POT1 que se une a las repeticiones teloméricas monocatenarias, TIN2 y TPP1 que conectan POT1 a TRF1 y TRF2 y RAP1 que interacciona con TRF2. Figura adaptada de [37,44].

Los telómeros se acortan en cada ciclo de división celular como resultado de la incompleta replicación de los cromosomas lineales, fenómeno conocido como "problema de la replicación terminal del ADN". La ADN polimerasa es incapaz de replicar el ADN en dirección 3'→5' dando como resultado la replicación incompleta en el extremo 5' de la hebra discontinua. En ausencia de un mecanismo compensatorio los telómeros pierden aproximadamente 50-200 nucleótidos en cada división celular, produciéndose en cada ciclo de replicación un acortamiento de la LT hasta alcanzar una longitud crítica en la cual la estructura de bucle-t ya no es estable. En esta instancia, los extremos de los telómeros son reconocidos por la maquinaria de reparación del ADN como rotura del ADN de doble cadena lo que resulta en la activación de la DDR y finalmente desencadena la senescencia replicativa [28,45–47]. Aunque esta es la principal razón del acortamiento telomérico, otros factores pueden afectar la velocidad de dicho acortamiento. Se ha observado un acelerado acortamiento telomérico en respuesta al estrés oxidativo [48,49]. Las regiones teloméricas son más sensibles que el resto del cromosoma a las modificaciones oxidativas debido a su alto contenido en guaninas [50]. En condiciones de estrés oxidativo moderado, las roturas de cadena simple se acumulan preferentemente en las regiones teloméricas lo que resulta en una incompleta replicación de los telómeros y por lo tanto un acortamiento acelerado [51,52].

Telomerasa

Para resolver el problema de la replicación terminal, las células utilizan un mecanismo independiente de la replicación, que consiste en la adición de repeticiones teloméricas al extremo protuberante de los cromosomas mediante una enzima llamada telomerasa. Originalmente descubierta por Carol Greider y Elizabeth Blackburn en el ciliado *Tetrahymena thermophila* [53], la telomerasa es un complejo ribonucleoproteico compuesto por una subunidad catalítica proteica, denominada TERT (Telomerase Reverse Transcriptase) y un componente de ácido ribonucleico (ARN), conocido como TERC (Telomerase RNA component). Aunque TERT y TERC son suficientes para producir actividad telomerasa *in vitro*, *in vivo* se requieren además otras cuatro proteínas (disquerina, HP2, NOP10, GAR1). Estas proteínas forman un complejo que se asocia a TERC y son esenciales para la estabilidad, localización subcelular y el funcionamiento del complejo telomerasa [54] (Figura 3).

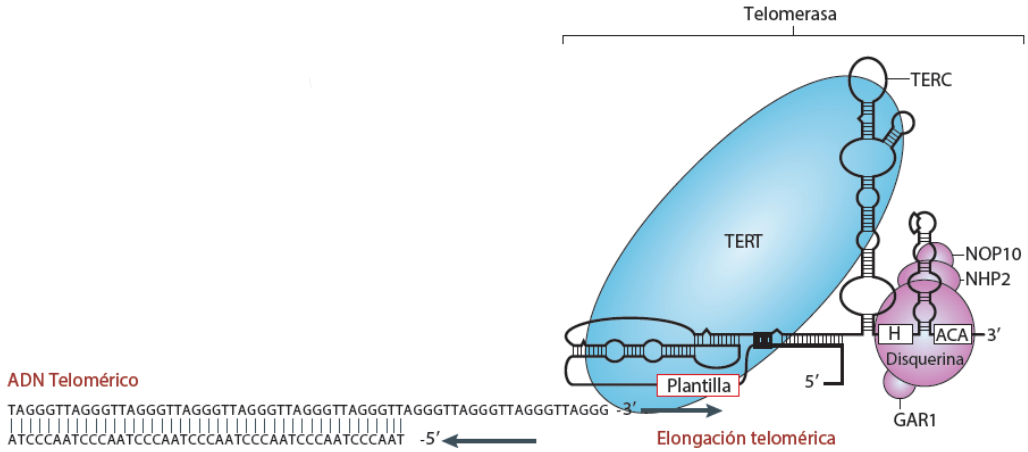

Figura 3 | Estructura de la telomerasa. El complejo telomerasa está formado por una proteína con actividad de transcriptasa reversa (TERT), un componente de ARN (TERC) y cuatro proteínas accesorias (disquerina, NHP2, NOP10 y GAR1). TERC contiene una secuencia conservada que TERT utiliza como molde para la síntesis de nuevas repeticiones telómericas. Figura adaptada de [37].

La síntesis de nuevas repeticiones teloméricas implica la transcripción inversa, catalizada por TERT, de una secuencia conservada del componente de ARN de la telomerasa. Por lo tanto, la función de la telomerasa es análoga a las retrotranscriptasas virales [55]. La elongación del telómero por la telomerasa se realiza en tres pasos. En el primer paso se produce el reconocimiento y unión de la telomerasa al extremo protuberante 3’ del telómero. La secuencia conservada de TERC es parcialmente complementaria al extremo telomérico lo que permite la formación de un híbrido ADN-ARN. En el siguiente paso TERT incorpora nucleótidos al extremo protuberante del telómero utilizando la secuencia conservada de TERC como molde. En el último paso se produce la disociación y translocación de la telomerasa al nuevo extremo 3' del ADN

telomérico para la siguiente ronda de síntesis. Finalmente, tras varias rondas de síntesis, la hebra complementaria rica en citosinas es sintetizada por la ADN polimerasa (Figura 4).

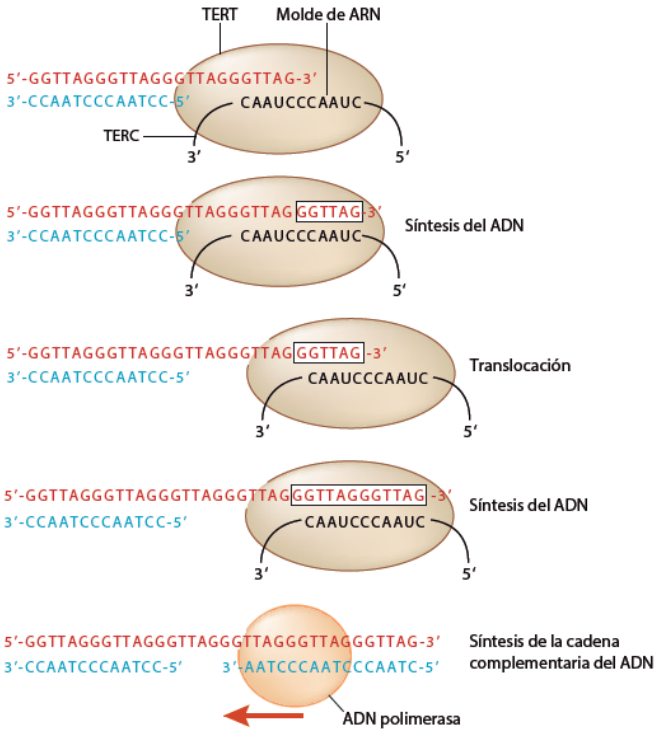

Figura 4 | Elongación del telómero por la telomerasa. La telomerasa agrega repeticiones teloméricas al extremo protuberante 3′ del telómero. La subunidad TERT con actividad de transcriptasa reversa utiliza la secuencia conservada de TERC como molde para incorporar nucleótidos al ADN telomérico. Una vez que la síntesis de ADN ha llegado al final de la secuencia conservada, la telomerasa se transloca y se realiza una nueva ronda de síntesis. El proceso de elongación se completa cuando la ADN polimerasa sintetiza la cadena complementaria. Figura adaptada de [56].

La telomerasa se expresa en las células germinales, en células madre adultas y en algunas células somáticas proliferativas como los linfocitos. En la mayoría de las células somáticas la actividad telomerasa es nula, debido a la represión transcripcional de la subunidad catalítica TERT. En contraste con las células germinales, en las cuales la actividad telomerasa es alta y por lo tanto suficiente para el mantenimiento de la LT, la actividad telomerasa en las células madre adultas y en los linfocitos no es suficiente para contrarrestar el acortamiento progresivo producido con cada división celular [12,56]. Por lo tanto, estas células solo pueden dividirse una cantidad limitada de veces; cuando los telómeros se acortan más allá de un punto crítico, el cromosoma se torna inestable lo que activa la DDR y finalmente la senescencia replicativa (Figura 5).

Senescencia celular

La senescencia celular se define como una detención permanente e irreversible del ciclo celular en respuesta al estrés. Este mecanismo fue descrito en 1961 por Hayflick y Moorhead, cuando observaron que los fibroblastos humanos podían dividirse solamente un número finito de veces en cultivo [58]. Posteriormente se ha demostrado que la limitada capacidad proliferativa, conocida como senescencia replicativa, se produce como consecuencia del acortamiento progresivo de los telómeros tras cada división celular [17] y que la expresión

ectópica de la enzima telomerasa contrarresta el acortamiento telomérico, y prolonga la capacidad proliferativa de las células [59].

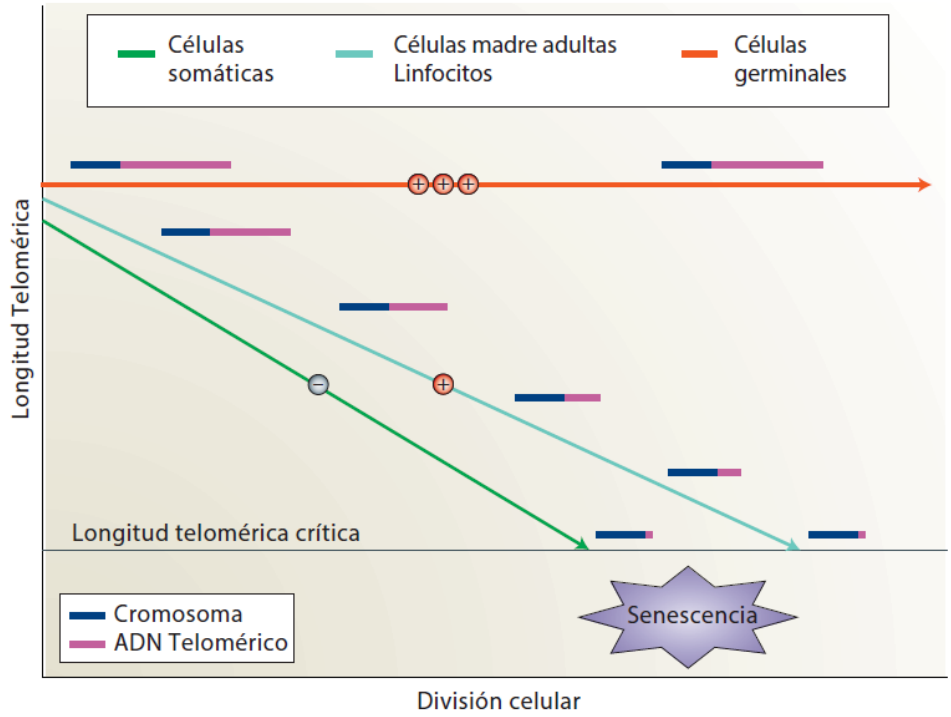

Figura 5 | Longitud telomérica, actividad telomerasa y senescencia. La telomerasa es la enzima responsable del mantenimiento de la longitud de los telómeros. Las células germinales son capaces de mantener la longitud telomérica estable debido a que presentan una alta actividad telomerasa. La mayoría de las células somáticas presentan una actividad telomerasa nula, por lo tanto el telómero se acorta en cada división celular como consecuencia del problema de la replicación terminal. En el caso de las células madres adultas y en algunas células somáticas proliferativas como los linfocitos la actividad telomerasa es baja, por lo que no es suficiente para contrarrestar el acortamiento telomérico producido en cada división celular. Este acortamiento progresivo conduce a telómeros críticamente cortos, lo que desencadena la respuesta de daño al ADN y finalmente la senescencia replicativa. Figura adaptada de [57].

Las células senescentes se caracterizan por presentar cambios en la expresión génica, un fenotipo secretor alterado caracterizado por la liberación de citoquinas proinflamatorias y quimioquinas y la adquisición de cierta resistencia a la apoptosis.

Envejecimiento del sistema inmune

A medida que un individuo envejece, el sistema inmune experimenta una serie de cambios, conocidos como inmunosenescencia, que se asocian a una disminución de la función inmune. Estos cambios en la función inmune ocurren como consecuencia de alteraciones tanto en el sistema inmune innato como adaptativo y contribuyen a la mayor susceptibilidad de las personas de edad avanzada a las infecciones, al cáncer, a las enfermedades autoinmunes y a una menor respuesta a la vacunación. La inmunosenescencia se caracteriza por (i) la disminución de la capacidad de autorrenovación de las células madre hematopoyéticas en la médula ósea,

(ii) la involución del timo, (iii) la disminución de los linfocitos T naïve circulantes, (iv) el aumento de los linfocitos T de memoria CD28- CD57+ con limitado potencial proliferativo, (v) el aumento de los niveles de citoquinas proinflamatorias y (vi) una disminución del cociente de linfocitos T CD4/CD8 [60].

Estudios longitudinales en ancianos han demostrado que la inversión del ratio CD4/CD8 (<1.0) se asocia a un incremento de la mortalidad en individuos mayores de 80 años [61,62]. Además, en estos estudios la disminución de la proliferación de células T y el incremento de células T CD28- también resultaron ser predictores de morbilidad y mortalidad. La inversión del ratio se produce como resultado de una pobre respuesta de las células T a mitógenos y una acumulación de células T CD8+ altamente diferenciadas debido a la expansión de las mismas en respuesta a antígenos. Estas células altamente diferenciadas han alcanzado la senescencia replicativa por lo cual presentan telómeros cortos y una marcada disminución de la capacidad proliferativa, y se caracterizan por la pérdida de la expresión de la molécula co-estimuladora CD28 y un aumento de la expresión del marcador de la historia proliferativa CD57 (Figura 6). Las células CD8+ senescentes se mantienen viables y activas metabólicamente por largos períodos de tiempo y además adquieren un fenotipo secretor proinflamatorio y resistencia a la apoptosis [63].

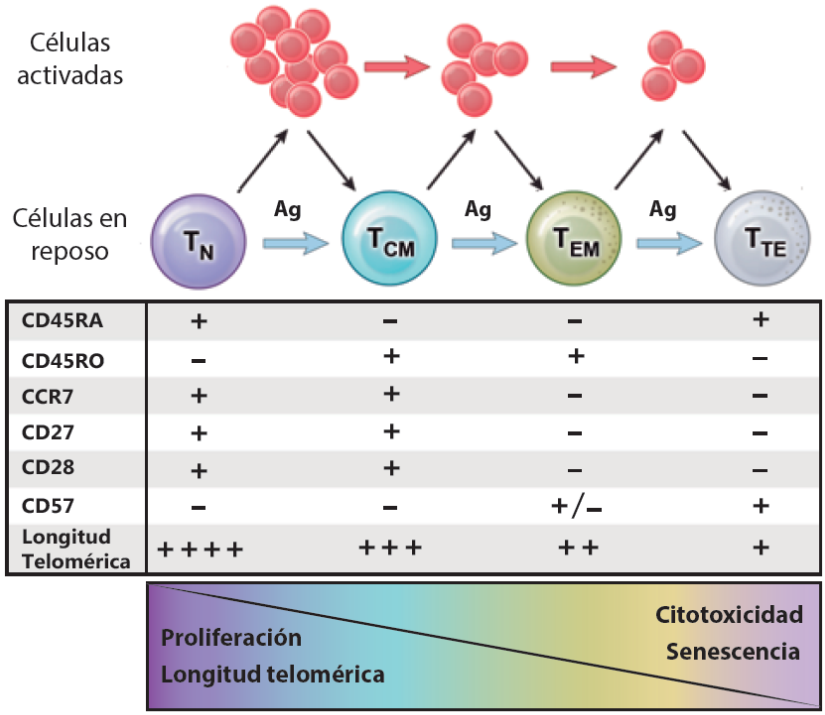

Figura 6 | Diferenciación de las células T. La estimulación antigénica induce la diferenciación de las células T naïves hacia células terminalmente diferenciadas. El proceso de maduración se caracteriza por un progresivo acortamiento de la longitud telomérica y una disminución de la capacidad proliferativa como así también por un aumento de la función efectora y de la senescencia. La expresión de los marcadores celulares de superficie permite identificar las diferentes subpoblaciones celulares. T_N:célula T naïve, T_{CM}: célula T de memoria central, T_{EM}: célula T de memoria efectora, T_{TE}: célula T terminalmente diferenciada, Ag: antígeno. Figura adaptada de [64].

Debido a que la exposición a antígenos y la inflamación resultan en la acumulación gradual de las células senescentes CD8+ CD28- CD57+, no es sorprendente que las infecciones virales crónicas, tales como el VIH, estén asociadas con una expansión progresiva de esta subpoblación celular.

Longitud telomérica e infección por VIH

Estudios observacionales han demostrado que las personas VIH+ tienen una LT medida en sangre total más corta que sus pares no infectados [13,15,16,45]. Al igual que en población general, la edad se asocia inversamente a la LT en la población VIH+. En cuanto a los factores relacionados con el VIH, tales como la duración y la severidad de la progresión de la infección, un estudio realizado en pacientes naïve determinó que la carga viral ≥ 100.000 copias/ml se asocia significativamente a menor LT [65]. Sin embargo, en pacientes en TAR existe cierta controversia. Zanet *et al* [13] han determinado que únicamente una carga viral ≥ 100.000 copias/ml antes del inicio de TAR se asocia al acortamiento telomérico, por el contrario en el estudio de Liu *et al* [16] se observa que tanto la duración de la infección (estimada como el tiempo desde el diagnóstico) como el bajo recuento de nadir CD4 (≤ 350 células/µl) se asocian de forma significativa a menor LT.

Recientemente, dos estudios han determinado que el acortamiento telomérico ocurre rápidamente tras la seroconversión [66,67]. En el trabajo de González-Serna *et al* se observó, en un grupo de 51 usuarios de drogas inyectables, una disminución del 13 % de la LT a los 3 meses de la seroconversión. Asimismo, Leung *et al*, tras determinar la LT en 31 usuarios de drogas inyectables seroconversores, estimaron que la tasa del acortamiento telomérico es de 650 pares de bases por año durante los dos primeros años tras la seroconversión. Esta tasa de acortamiento es aproximadamente 26 veces superior al acortamiento de 25 pares de bases al año observado en la población general [68].

El acortamiento telomérico debido a la infección por VIH ocurre principalmente en la subpoblación de células T CD8+. Un estudio ha determinado que, comparado con la población no VIH, el acortamiento de la LT en sangre de la población VIH+ es mayor en las células T CD8+ que en las CD4+ [45]. Además, en otro trabajo se ha observado que durante la infección por VIH no tratada, la LT de la subpoblación de células T CD8+ CD28- de individuos de mediana edad es similar a la LT en células mononucleares de sangre periférica (PBMCs) de individuos centenarios [63].

Un estudio en el que se ha comparado la LT de individuos VIH+ y no VIH, se estimó que el efecto de la infección por VIH determinada en sangre es similar a la observada en población no VIH tras una década de envejecimiento [13]. Asimismo, el envejecimiento acelerado causado por el VIH se ha observado en estudios que utilizaron un biomarcador de envejecimiento diferente a la LT en sangre. Mediante la utilización de la metilación del ADN, dos estudios han estimado que la infección por VIH resulta en un incremento de la edad biológica de aproximadamente 5 años en relación con la edad cronológica [7,8].

Múltiples mecanismos pueden contribuir al acortamiento telomérico que se produce en la infección por VIH: (i) Inhibición de la actividad telomerasa por proteínas del VIH, (ii) la inmunosenescencia producida por la infección del VIH y (iii) la inhibición de la telomerasa por los N(t)RTIs.

Inhibición de la telomerasa por el VIH

Como se ha mencionado anteriormente, la actividad telomerasa es nula en la mayoría de las células somáticas debido a la represión transcripcional de la subunidad catalítica TERT. Sin embargo los linfocitos T expresan la proteína TERT tanto en células T en reposo, que no tienen actividad telomerasa detectable, como en timocitos y células T activadas que sí presentan actividad telomerasa. Por lo tanto, la regulación de la actividad telomerasa en las células T se debe a un mecanismo post-traduccional. Se ha demostrado que la activación de las células T da como resultado la fosforilación de la proteína TERT y su translocación del citoplasma al núcleo [69].

Se ha observado que el VIH inhibe la actividad telomerasa en PBMCs tanto *in vitro* como *in vivo* [70]. Además, en las células CD4+ infectadas por VIH, la disminución de la actividad telomerasa se asocia a menores niveles de la proteína TERT fosforilada [71]. Recientemente Comadini *et al* han demostrado que la proteína Tat del VIH está implicada en la reducción de la fosforilación de la proteína TERT y de su posterior importación al núcleo celular [72].

Inmunosenescencia e infección por VIH

La activación inmune crónica producida por la infección por VIH se traduce en cambios en el sistema inmune similares a los observados en la población de avanzada edad. En ambos casos se observa una disminución de la función tímica, inversión de ratio CD4/CD8, una pérdida de la capacidad regenerativa de células T y por tanto una reducción de células naïve y un incremento de las células T CD8+ senescentes. Como se mencionó anteriormente, las células T senescentes son células altamente diferenciadas con escasa capacidad proliferativa y telómeros cortos que

se caracterizan por la pérdida de la expresión de CD28 y un aumento de la expresión de CD57. En el caso de la infección por VIH no tratada la subpoblación de células T CD8+ CD28- representa aproximadamente el 65% del total de las células T CD8+ [73], lo cual es similar al 60% detectado en la población adulta octogenaria no VIH [74]. Además, la infección por VIH se asocia a niveles elevados de inflamación que permanecen elevados a pesar del TAR efectivo. Esta inflamación persistente durante el tratamiento podría deberse a una serie de factores, incluida la replicación residual del VIH en los reservorios, la coinfección por otros virus que también pueden tener un estado de replicación crónica (citomegalovirus, virus de la hepatitis B y C) y la translocación bacteriana. Por lo tanto, existe una estrecha relación entre activación inmune, inflamación e inmunosenescencia (Figura 7).

El TAR revierte parcialmente la inmunosenescencia asociada al VIH. Tras el control de la replicación viral se produce un incremento de las subpoblaciones de linfocitos T naïve y de memoria central. Estas subpoblaciones celulares menos diferenciadas se caracterizan por tener telómeros más largos que las células senescentes. Un estudio longitudinal determinó que tras el inicio del TAR se produce un incremento de la LT en la población de células T que se correlaciona principalmente con cambios de las subpoblaciones CD8+ hacia fenotipos menos maduros [75].

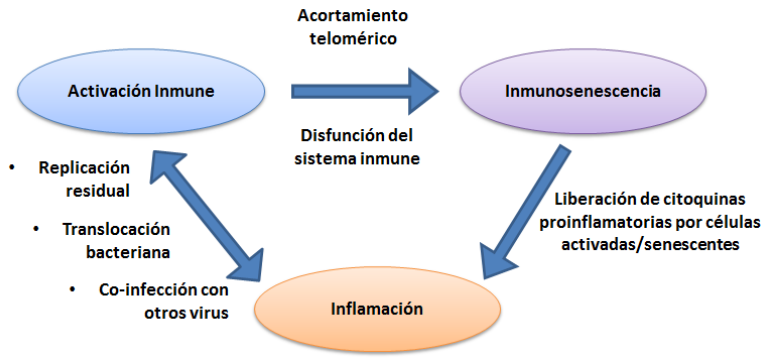

Figura 7 | Relación entre inflamación, activación inmune y senescencia

Inhibición de la telomerasa por los N(t)RTIs

Como se explicó anteriormente, la subunidad TERT de la telomerasa presenta similitudes estructurales y funcionales con la transcriptasa inversa del VIH [55]. Basados en dicha similitud, diversos estudios se han llevado a cabo con el fin de determinar si los fármacos inhibidores de la transcriptasa inversa pueden inhibir también la actividad de la telomerasa celular.

Los N(t)RTIs son fármacos que, tras activarse mediante fosforilación en el interior celular, inhiben la actividad de la transcriptasa inversa del VIH debido a que compiten con los nucleótidos fisiológicos. Estos fármacos carecen de un grupo OH en el carbono 3' de la ribosa

por lo que actúan como terminadores de cadena. Cuando la transcriptasa inversa del VIH incorpora un N(t)RTI al ADN viral naciente se bloquea la incorporación del siguiente nucleótido impidiendo que se sintetice la totalidad del ADN vírico.

Estudios *in vitro* han determinado que los fármacos zidovudina (AZT), estavudina (d4T), didanosina (ddI) y abacavir (ABC) inhiben la actividad telomerasa causando acortamiento telomérico [76–80]. La inhibición se produce porque estos fármacos son análogos de los nucleósidos celulares timidina (T), adenina (A) y guanosina (G) (Tabla 1), de esta manera cuando la telomerasa incorpora alguno de estos N(t)RTIs a las repeticiones teloméricas TTAGGG el proceso de elongación del telómero queda bloqueado. En contraste, no se observa inhibición de la actividad telomerasa cuando se utilizan fármacos inhibidores de la transcriptasa inversa no análogos de nucleósidos (NNRTIs) como son nevirapina ó efavirenz, incluso en presencia de altas concentraciones de los mismos [76].

Un trabajo más reciente ha estudiado el impacto sobre la actividad telomerasa de los N(t)RTIs más utilizados en el TAR actual, tales como tenofovir difumarato (TDF), lamivudina (3TC) y emtricitabina (FTC), como así también de los previamente estudiados AZT y ABC [81]. En este estudio se ha determinado que TDF es un inhibidor de la telomerasa más potente que ABC o AZT, ya que es el único que inhibe significativamente la actividad telomerasa a concentraciones terapéuticas; y además, se observó que dicha inhibición se asociaba a una disminución de la LT. Por otro lado, también se determinó que altas concentraciones de 3TC y FTC inhiben la actividad telomerasa. Este resultado es sorprendente porque tanto 3TC como FTC son análagos de citidina (C) por lo que la inhibición de la telomerasa no se explica por la incorporación de estos fármacos a las repeticiones teloméricas, sino que deben estar implicados otros mecanismos.

N(t)RTIs	Análogo del nucleós(t)idos
AZT	T
d4T	T
ddI	A
TDF	A
ABC	G
FTC	C
3TC	C

Tabla 1 | Fármacos N(t)RTIs y nucleós(t)idos fisiológicos

A pesar de la demostrada inhibición de la telomerasa *in vitro* causada por algunos N(t)RTIs y de su asociación con el acortamiento telomérico, hasta la fecha se han publicado pocos trabajos que evalúan *in vivo* los efectos de los N(t)RTIs sobre la LT [13,15,81,82], por lo que aún no se ha

determinado cuál es su impacto *in vivo* tanto en pacientes con la carga viral suprimida como en aquellos pacientes que empiezan TAR de primera línea.

Objetivos

1. Determinar si los N(t)RTIs a concentraciones terapéuticas inhiben la actividad telomerasa celular *in vitro*.

2. Estudiar el impacto *in vivo* de los N(t)RTIs en la LT en sangre de pacientes VIH+ con TAR efectivo y prolongada supresión virológica.

3. Estudiar el impacto *in vivo* de los N(t)RTIs en la LT en sangre en pacientes naïve que comienzan TAR de primera línea.

Metodología y Resultados

Publicación 1

DIFFERENTIAL EFFECTS OF TENOFOVIR, ABACAVIR, EMTRICITABINE AND DARUNAVIR ON TELOMERASE ACTIVITY IN VITRO

Como se ha explicado en la introducción, en población general existe una estrecha relación entre el acortamiento telomérico en sangre y diversas patologías asociadas al envejecimiento. En el caso del paciente VIH, a pesar del éxito del TAR, dichas patologías se observan a edades menos avanzadas. Además, diversos estudios han demostrado que el paciente VIH presenta una menor LT que la observada en población general. Dada la similitud estructural y funcional entre la transcriptasa reversa del VIH y la telomerasa celular, cabe pensar que un posible factor implicado en el envejecimiento prematuro del paciente VIH podría ser el acortamiento telómerico debido a la inhibición de la actividad telomerasa por los fármacos N(t)RTIs.

En este trabajo se ha estudiado el efecto in vitro del tratamiento de PBMCs con concentraciones terapéuticas de tenofovir (TFV), compuesto activo de TDF y de los fármacos antirretrovirales ABC, FTC y darunavir (DRV) sobre la actividad telomerasa. Los resultados obtenidos indican que TFV y ABC inhiben la actividad telomerasa a concentraciones terapéuticas in vitro, siendo TFV el fármaco que produce mayor inhibición. Además, la inhibición se produce a nivel de la actividad enzimática, no de los niveles de proteína o la expresión génica.

Mi contribución personal en este trabajo consistió en el diseño y realización de la parte experimental (tratamiento de PBMCs con fármacos antirretrovirales, cuantificación de la actividad telomerasa, determinación de los niveles de TERT y determinación de los niveles de expresión de los genes del complejo telomerasa y shelterina). Además, he participado activamente en el análisis de los resultados y en la elaboración del manuscrito.

Differential Effects of Tenofovir, Abacavir, Emtricitabine, and Darunavir on Telomerase Activity In Vitro

Natalia Stella-Ascariz, MS, Rocío Montejano, MD,† Laura Pintado-Berninches, MS,‡ Susana Monge, MD,§ José I. Bernardino, MD,† Ignacio Pérez-Valero, MD,† María L. Montes, MD,† Jesús Mingorance, PhD,* Rosario Perona, PhD,‡ and José R. Arribas, MD†*

Abstract: In vitro, tenofovir and abacavir induced a significant dose-dependent inhibition of telomerase activity at therapeutic concentrations in peripheral blood mononuclear cells of healthy subjects. Median inhibition of telomerase activity by tenofovir at 0.5 and 1 μM was 29% [Interquartile range (IQR) 29%–34%, $P = 0.042$] and 28% (IQR 28%–41%, $P = 0.042$), respectively. Abacavir inhibition was 12% (IQR 9%–13%, $P = 0.043$) at 3 μM and 14% (IQR 10%–29%, $P = 0.043$) at 10 μM. Tenofovir and abacavir did not change human telomerase reverse transcriptase (hTERT) levels or mRNA levels of other telomerase complex genes. Exposure to emtricitabine or darunavir did not affect telomerase activity, hTERT protein levels, or mRNA levels of telomerase/shelterin genes.

Key Words: HIV infection, antiretroviral therapy, telomerase, telomere, hTERT

(*J Acquir Immune Defic Syndr* 2017;74:91–94)

INTRODUCTION

There is growing concern about the issue of aging of HIV-infected patients. It is well established that HIV-infected patients have an increased risk for several "non-AIDS" complications (cardiovascular disease, malignancy, liver disease, kidney disease, bone disease, and neurocognitive decline) that are classically associated with the normal aging process.[1] It remains unclear if the higher risk of these complications is expression of an "accelerated" aging process, complications occurring at earlier ages, or of an "accentuated" aging process—higher prevalence of complications at every age strata.[2,3] It is also unknown if this accentuated or accelerated aging is caused by the proinflammatory state associated with even well-controlled HIV infection, traditional risk factors (such as smoking) that are more prevalent among HIV-infected people, or other still unknown causes.[4]

Another potential cause of accelerated or accentuated aging in HIV-infected patients could be telomere shortening caused by antiretroviral drugs.[5] There is a close association between shortened telomere length (TL) in peripheral blood mononuclear cells (PBMCs) and diseases of aging, including increased cardiovascular diseases and dementia.[6,7]

Telomerase is a ribonucleoprotein enzyme complex with a RNA template (TERC), a human telomerase reverse transcriptase (hTERT) subunit, and other regulatory proteins that together with the shelterin complex maintains telomere structure.[8] Telomerase adds repetitive TTAGGG sequences to the ends of chromosomes, compensating for the progressive telomeric loss occurring at each cell division. Because of structural and mechanistic similarity to HIV reverse transcriptase, nucleoside/nucleotide reverse transcriptase inhibitors [N(t)RTI] can inhibit telomerase.[9] Zidovudine (AZT), stavudine (d4T), didanosine (ddI), and abacavir (ABC) can inhibit telomerase activity in replicating cell lines in vitro, leading to accelerated shortening of TL.[10,11] This inhibition is not observed with nonnucleoside reverse transcriptase inhibitors.[12] Recently, Leeansyah and collaborators reported that tenofovir (TFV)[13] at therapeutic concentrations is a potent inhibitor of telomerase

Received for publication May 24, 2016; accepted July 29, 2016.

From the *Microbiology Service, Hospital Universitario La Paz, IdiPAZ, Madrid, Spain; †HIV Unit, Internal Medicine Service, Hospital Universitario La Paz, IdiPAZ, Madrid, Spain; ‡Instituto de Investigaciones Biomédicas CSIC/UAM, IdiPAZ, Biomarkers and New Therapies and CIBER de Enfermedades Raras (CIBERER), Madrid, Spain; and §Universidad de Alcalá de Henares, Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBERESP), Madrid, Spain.

Supported by Grant PI13/01467 and PI14/01495 from Fondo de Investigaciones Sanitarias (supported by FEDER funds). Instituto de Salud Carlos III.

Presented as a poster in the 23th Conference on Retrovirus and Opportunistic Infections, February 22–25, 2016, Boston, MA.

N.S. is supported by a predoctoral fellowship from Fondo de Investigaciones Sanitarias. R.M. is supported by a Río Hortega fellowship from Fondo de Investigaciones Sanitarias. J.I.B. has received payment for lectures including service on the speaker's bureau from ViiV Healthcare and Janssen and currently received payment for expert testimony from Gilead Sciences and MSD. I.P.V. is supported by a Grant from Instituto de Salud Carlos III and Gilead Sciences and he has received payment for consultancy and payment for lectures including service on the speaker's bureau from Janssen Cilag, Gilead and VIIV and Gilead Sciences, Janssen Cilag, VIIV, BMS and MSD, respectively. M.L.M. is currently receiving payment for board membership and for development of educational presentations from Janssen and Abbvie and Gilead, respectively. Also, she has received payment for consultancy from Janssen, Abbvie, ViiV, for expert testimony from Abbvie, Brsitol, Janssen and for lectures including service on the speaker's bureau from Bristol, Janssen, ViiV, Abbvie. J.R.A. is currently receiving payment for board membership and for consultancy from Viiv, Janssen, Abbvie, BMS, Gilead, MSD and Viiv, Janssen, BMS, Gilead, and MSD, respectively. Also, he is receiving payment for lectures including service on the speaker's bureau from Viiv, Janssen, Abbvie, BMS, Gilead, and MSD. The remaining authors have no funding or conflicts of interest to disclose.

R.P. and J.R.A. equal contribution to this manuscript.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.jaids.com).

Correspondence to: José R. Arribas, MD, PhD, Consulta Medicina Interna 2, Hospital La Paz, IdiPAZ, Castellana 261, 28046 Madrid, Spain (e-mail: joser.arribas@salud.madrid.org).

activity, causing telomere shortening in vitro. In Leeansyah et al study, lamivudine (3 TC) and emtricitabine (FTC) also inhibited telomerase activity although only at high concentrations. This finding is surprising because, being cytidine analogues, 3 TC and FTC should not act as chain terminators and therefore other mechanism of telomerase inhibition by 3 TC and FTC could be operating.

Two studies have reported that saquinavir, a protease inhibitor (PI), is able to increase telomerase activity in PBMCs[14,15] and demonstrated that telomerase up-regulation appeared to be the result of enhanced expression of hTERT in human T leukemia cells in vitro.[16] Saquinavir is no longer a preferred protease inhibitor in expert guidelines. Darunavir (DRV) boosted with ritonavir is the recommended protease inhibitor in the majority of expert guidelines. The impact of DRV on telomerase activity is currently unknown.

The main objective of our study was to confirm if ABC, FTC and especially TFV, at therapeutic concentrations, inhibit telomerase activity in vitro in activated-PBMCs. Secondly, we assayed if DRV is able to increase telomerase activity in vitro. Finally, we wanted to evaluate the possible impact of these antiretrovirals on mechanisms of telomerase inhibition different from chain termination such as expression of telomerase genes.

METHODS

Culture of PBMCs In Vitro With Antiretrovirals

PBMCs from healthy volunteers were isolated from whole blood by density gradient centrifugation using Ficoll-Pague PLUS (GE Healthcare). PBMCs were cultured in RPMI medium with 10% FBS supplemented with phytohemaglutinin, M form (PHA-M) (1% vol/vol; Gibco—Life Technologies), and human recombinant interleukin 2 (IL-2) (5 ng/mL; Gibco—Life Technologies) for 72 hours. Subsequently, 4×10^6 activated PBMCs were treated with increased concentrations of TFV, ABC, FTC or DRV (National Institute of Health AIDS Research and Reference Reagent Program) in fresh medium plus PHA-M/IL-2 for another 72 hours. Concentrations used for ABC and FTC were 0; 1; 3; 10; 50; and 100 μM, for TFV were 0; 0.1; 0.5; 1; 3; and 5 μM and for DRV were 0; 0.5; 1; 5; 10; 50 μM. Concentrations were calculated based on the pharmacokinetic parameters summarized in http://www.hiv-druginteractions.org/fact_sheets website of University of Liverpool.

Measurement of Telomerase Activity

We determined telomerase activity according to the Telomeric Repeat Amplification Protocol (TRAP) by TRAPeze Telomerase Detection kit (EMD Millipore, Billerica, MA) using radioisotopic detection according to the manufacturer's instructions with following modifications: radioactive end-labeling of the TS Primer[17] was incubated 30 minutes at 37°C and 10 minutes at 85°C, telomerase extension reaction was performed with 3 serial dilutions of cell extract at 30°C for 30 minutes followed by 5 minutes denaturation at 94°C, and amplification of the telomeric repeats was done in 30 cycles. The reaction was visualized by autoradiography and was analyzed using the image-processing program ImageJ (http://imagej.nih.gov/ij/). Telomerase activity was normalized using the internal control provided in the kit and expressed relative to untreated PBMCs.

Western Blot Analysis of hTERT Expression

20 μg of total cellular protein was subjected to 8% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore). Membranes were then blocked with 5% milk and incubated with rabbit anti-hTERT antibody (1:1000) (cat no. sc-7212; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, followed by incubation with a secondary anti-rabbit antibody (1:1000) conjugated with horseradish peroxidase at room temperature for 30 minutes. Membranes were rehybridized with α-tubulin antibody as a housekeeping expressed control protein. Membrane antibody binding was detected by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology). Image quantifications were performed using ImageJ software.

Measurement of Gene Expression of the Telomerase/Shelterin Complex

Total RNA was isolated from cells using Tri Reagent (Sigma) and was quantified spectrophotometrically. First-strand cDNA was synthesized from 1 μg of total RNA by reverse transcriptase in a volume of 20 μL containing 200 U of Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega), 1X M-MLV 5X reaction buffer (Promega), 20 U of RNase OUT (Invitrogen), 0.5 μg random primer (Promega), and 0.5 mM dNTPs. The reaction was performed for 60 minutes at 37°C and cDNA was stored at −80°C until use.

The mRNA expression level of the genes coding for the different telomerase or shelterin complex subunits (*hTERT*, *TERC*, *DKC1*, *TINF2*, *TRF1* and *TRF2*) was quantified by quantitative real-time PCR (qPCR) using TaqMan Gene Expression Assay (Applied Biosystem). To normalize the amount of total mRNA present in each reaction, we amplified an endogenous housekeeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). qPCR was performed with 100 ng of cDNA in a total volume of 20 μL containing 1X TaqMan Universal Master Mix II, no UNG and 1X TaqMan Gene Expression Assay of each gene (Applied Biosystem) (see Table 1, Supplemental Digital Content, http://links.lww.com/QAI/A880). Samples were performed in duplicate using a Real Time Stratagene MX3000P. The thermal cycling conditions included preincubation for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C.

Statistical Analysis

Statistic evaluation was performed with STATA v12 (Stata Corporation, College Station, Texas). Statistical significance for telomerase activity, levels of hTERT protein, and expression of telomerase complex subunits genes were calculated using

Wilcoxon signed-rank test. P values < 0.05 were considered statistically significant.

The institutional review board of Hospital La Paz approved the study and samples were obtained after written informed consent.

RESULTS

TFV and ABC Inhibit Telomerase Activity In Vitro

We analyzed the effect of 3 N(t)RTIs (TFV, ABC and FTC) and 1 PI (DRV) on telomerase activity in PHA-activated PBMCs from healthy volunteers after 72 hours of treatment. We performed 5 independent experiments with all N(t)RTIs and 6 independent experiments with DRV. Telomerase activity was expressed relative to untreated PBMCs.

Of the N(t)RTIs tested, only TFV and ABC induced a significant dose-dependent decrease of telomerase activity within the therapeutic concentration range. Median inhibition induced by TFV at 0.5 and 1 μM was 29% (IQR: 29%–34%; range: 12%–39%; $P = 0.042$) and 28% (IQR: 28%–41%; range: 25%–47%; $P = 0.042$), respectively. For ABC at 3 and 10 μM, median inhibition was 12% (IQR: 9%–13%; range: 8%–17%; $P = 0.043$) and 14% (IQR: 10%–29%; range: 7%–40%; $P = 0.043$), respectively. Exposure to FTC or DRV did not affect telomerase activity even at concentrations above the therapeutic plasma level range (Fig. 1A).

No Changes in Levels of hTERT Protein or Expression of the Telomerase/Shelterin Complex Genes.

Experiments were performed to establish whether reduction of telomerase activity mediated by TFV and ABC was the consequence of a decrease in the amount of hTERT protein. We determined the levels of hTERT after treatment for 72 hours with ABC, FTC and DRV (4 experiments each) and TFV (5 experiments). We did not find differences in the amount of hTERT between untreated and treated PBMCs (Fig. 1B). In addition, we measured the expression levels of the genes coding for the different telomerase and shelterin complex subunits (*hTERT*, *TERC*, *DKC1*, *TINF2*, *TRF1*, and *TRF2*). We measured the levels of mRNA after treatment for 72 hours with TFV, ABC, and DRV (6 experiments each) and FTC (5 experiments). We did not detect changes in expression of the genes that code for the catalytic subunit hTERT of the telomerase complex (Fig. 1C) or other telomerase subunits.

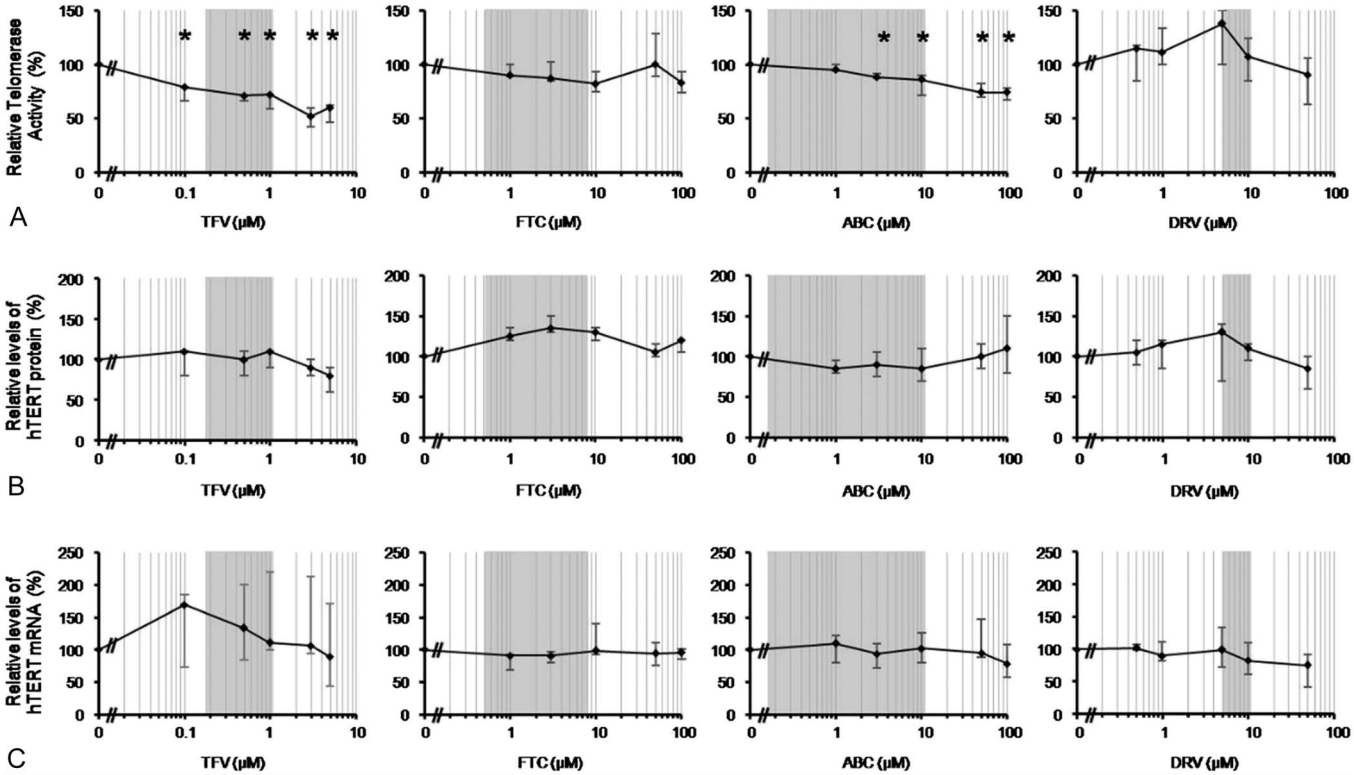

FIGURE 1. The effect of nucleos(t)ide reverse transcriptase inhibitors and darunavir on telomerase activity (A), levels of hTERT protein (B), and hTERT mRNA (C) in activated PBMCs. Closed circles represent the median, error bars represent interquartile range, and gray area represents the expected plasma levels in vivo for each antiretroviral (boosted DRV therapeutic range was assumed for DRV). The data are represented as percentage telomerase activity, levels of hTERT protein, and amount of hTERT mRNA of untreated cells. For telomerase activity (A), results are shown for TFV, ABC, and FTC (n = 5) and for DRV (n = 6). For levels of hTERT protein (B), results are shown for TFV (n = 5) and ABC, FTC, and DRV (n = 4). For levels of hTERT, mRNA results are shown for TFV, ABC, and DRV (n = 6) and FTC (n = 5). *$P < 0.05$.

DISCUSSION

In our study, we explored if N(t)RTIs could play a role in the aging process of HIV-infected patients by means of inhibition of telomerase activity and whether DRV could have a similar effect. We have found that TFV and ABC, but not FTC, produced a significant dose-dependent decrease of telomerase activity in PHA-activated PBMCs within the therapeutic concentration range in vivo. After 72 hours of treatment, telomerase inhibition caused by TFV was more than double the inhibition caused by ABC: 29% and 12%, respectively. The observed decrease in telomerase activity caused by TFV and ABC was not associated with a decrease in hTERT protein level, or a change in the expression of *hTERT* gene or the other genes that code for the subunits of the telomerase/shelterin complexes. Furthermore, we have shown that DRV did not affect telomerase activity, *hTERT* gene expression, or hTERT protein levels.

The active forms of TFV, ABC, and FTC compete with the intracellular dATP, dGTP and dCTP pools, respectively, and the incorporation of these nucleotide analogs causes viral DNA chain termination. Telomeres are made up of hexamer repeat TTAGGG sequences. Telomerase binds to the 3' end of the telomeres through its own RNA template and adds TTAGGG polynucleotides to the extreme, whereas the complementary strand is filled in by DNA polymerase later on. Consequently, inhibition of the reverse transcriptase activity of hTERT by the chain termination mechanism would be possible with TFV and ABC but not with FTC. Leeansyah and collaborators[13] have previously shown that TFV was the only N(t)RTI that at therapeutic concentrations in vitro significantly inhibited telomerase activity and enhanced shortening of TL. However, in their study, ABC and FTC were also able to inhibit telomerase activity at concentrations above the therapeutic range, but only ABC enhanced shortening of TL. Probably, our study underestimates the inhibition of activity telomerase with ABC and FTC because we performed measurements of telomerase activity after 72 hours of treatment with a single dose of each drug, in contrast to Leeansyah study in which N(t)RTIs were replenished every 48 hours. This difference could account for the lack of effect of FTC on telomerase activity observed in our study. Moreover, another limitation of our study is that we cannot determine whether the main effects of NRTIs were on $CD4^+$ or $CD8^+$ T cells, or specific subsets of T cells or non–T-cell population.

The in vivo relevance of the differential effects of TFV, ABC, FTC, and DRV on telomerase activity remains to be elucidated. Although Leeansyah and colleagues reported that in vivo telomere length was significantly inversely associated with the total duration of treatment with any N(t)RTI, other studies have not found this association. In a substudy of the MONET clinical trial[18] comparing darunavir/ritonavir monotherapy versus darunavir/ritonavir and 2 N(t)RTIs for maintenance of virological suppression, there were not significant differences between 2 arms after 3 years of follow-up in telomerase activity or mean change per year of telomere length.

Our results provide more evidence about the inhibition caused by some NRTI on telomerase activity. We have confirmed that TFV and ABC inhibit telomerase activity in activated PBMCs in vitro at therapeutic concentrations and that TFV is the most potent inhibitor. Telomerase activity inhibition caused by N(t)RTIs is probably due to inhibition of hTERT activity leading to chain termination and does not involve changes in expression levels of telomerase genes or hTERT protein. In addition, we did not find that DRV affects telomerase activity, *hTERT* gene expression, or hTERT protein levels. To our knowledge, ours is the first study showing a lack of implication of hTERT proteins levels and mRNA expression. The in vivo relevance of these findings remains to be elucidated.

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Publicación 2

IMPACT OF ANTIRETROVIRAL TREATMENT CONTAINING TENOFOVIR DIFUMARATE ON THE TELOMERE LENGTH OF AVIREMIC HIV-INFECTED PATIENTS

En la primera publicación presentada en esta tesis se ha determinado que TFV es el inhibidor más potente de la telomerasa *in vitro*. Dado que los datos sobre el impacto *in vivo* de los diferentes regímenes de TAR en el acortamiento telomérico era muy limitado y que las pautas preferentes de inicio de TAR incluyen TFV (administrado como TDF o tenofovir alafenamida), en este trabajo se ha estudiado el impacto de TDF en la longitud de los telómeros de los pacientes infectados por VIH que reciben TAR efectivo y presentan supresión virológica. Para ello se ha comparado la LT en una cohorte de pacientes VIH+ del Hospital Universitario La Paz entre un grupo de pacientes que estaba expuesto a TDF o lo había estado previamente y un grupo de pacientes que nunca estuvo expuesto a TDF. Los resultados obtenidos indican que en los pacientes VIH+ con prolongada supresión viral no se observan diferencias significativas en la LT en sangre entre el grupo de pacientes expuestos a TDF y el grupo de pacientes no expuestos a TDF.

Mi contribución personal en la parte experimental de este trabajo consistió en la recogida y procesamiento de las muestras de sangre de los pacientes de la cohorte, la adaptación de un protocolo previamente publicado de qPCR para la medición de la LT relativa y la determinación de la LT en sangre de los pacientes de la cohorte. En cuanto a la elaboración del manuscrito he participado en el análisis de los resultados obtenidos, el diseño de figuras y tablas y la escritura del artículo.

Impact of Antiretroviral Treatment Containing Tenofovir Difumarate on the Telomere Length of Aviremic HIV-Infected Patients

Rocio Montejano, MD, Natalia Stella-Ascariz, MS,† Susana Monge, MD,‡ José I. Bernardino, MD,* Ignacio Pérez-Valero, MD,* María L. Montes, MD,* Eulalia Valencia, MD,* Luz Martín-Carbonero, MD,* Victoria Moreno, MD,* Juan González-García, MD,* Francisco Arnalich, MD,* Jesús Mingorance, PhD,† Laura Pintado Berniches, MS,§ Rosario Perona, MD,§ and José R. Arribas, MD**

Objective: To evaluate the in vivo relevance of the inhibitory effect of tenofovir on telomerase activity observed in vitro.

Design: Cross-sectional study of HIV-infected patients with suppressed virological replication (HIV RNA <50 copies/mL for more than 1 year).

Methods: Telomere length in whole blood was measured by quantitative real-time polymerase chain reaction. We performed a multivariate analysis to elucidate variables associated with telomere length and also evaluated the association between telomere length and use of tenofovir difumarate (TDF) adjusted by significant confounders.

Results: 200 patients included, 72% men, median age 49 (IQR 45–54.5), 103 with exposure to a TDF containing antiretroviral treatment (ART) regimen (69.9% for more than 5 years) and 97 never exposed to a TDF containing ART regimen. In the multivariate analysis, significant predictors of shorter telomere length were older age ($P = 0.008$), parental age at birth ($P = 0.038$), white race ($P = 0.048$), and longer time of known HIV infection (10–20 and ≥20 years compared with <10 years, $P = 0.003$ and $P = 0.056$, respectively). There was no association between TDF exposure and telomere length after adjusting for possible confounding factors (age, parental age at birth, race, and time of HIV infection). Total time receiving ART and duration of treatment with nucleoside reverse transcriptase inhibitors were associated with shorter telomere length, but these associations were explained by time of known HIV infection.

Conclusions: Our data do not suggest that telomerase activity inhibition caused by TDF in vitro leads to telomere shortening in peripheral blood of HIV-infected patients.

Key Words: HIV infection, antiretroviral therapy, telomerase, telomere

(*J Acquir Immune Defic Syndr* 2017;76:102–109)

INTRODUCTION

HIV-infected patients have an increased risk for several "non-AIDS" complications such as cardiovascular disease, cerebrovascular events, malignancy, liver disease, kidney disease, bone disease, and neurocognitive decline that are classically associated with the normal aging process.[1]

There is a continuous debate about if the higher risk of these complications in HIV-infected patients is the expression of an "accelerated" aging process—complications occurring prematurely—, or an "accentuated" aging process—higher prevalence of complications at every age strata. The Danish HIV-cohort study has not found evidence to suggest accelerated aging in the HIV-infected population.[2] In contrast, 2 recent studies using epigenetic biomarkers of aging have found that HIV-infected patients have an age advancement of approximately 5 years compared with HIV uninfected controls.[3,4]

Proposed mechanisms for the abnormal aging of HIV-infected patients are the proinflammatory state and immune activation associated to even well-controlled HIV infection,[5] traditional risk factors (such as smoking) that are more prevalent among HIV-infected people, or other still unknown causes.

Another potential cause of accelerated or accentuated aging in HIV-infected patients could be telomere attrition.

Received for publication December 9, 2016; accepted February 20, 2017.

From the *HIV Unit, Internal Medicine Service, Hospital Universitario La Paz-IdiPAZ, Madrid, Spain; †Microbiology Service, Hospital Universitario La Paz-IdiPAZ, Madrid, Spain; ‡Universidad de Alcalá de Henares, Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBERESP), Madrid, Spain; and §Instituto de Investigaciones Biomédicas CSIC/UAM, IdiPAZ, Biomarkers and New Therapies and CIBER de enfermedades raras (CIBERER), Madrid, Spain.

Supported by grant PI13/01467 and PI14/01495 from Fondo de Investigaciones Sanitarias (supported by FEDER funds). Instituto de Salud Carlos III. N.S.-A. is supported by a predoctoral fellowship from Fondo de Investigaciones Sanitarias. R.M. is supported by a Río Hortega fellowship from Fondo de Investigaciones Sanitarias.

The authors have no conflicts of interest to disclose.

R.M. and N.S.-A. equally contributed to this manuscript.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.jaids.com).

Correspondence to: José R. Arribas, MD, PhD, Consulta Medicina Interna 2, Hospital La Paz, IdiPAZ, Paseo de la Castellana 261, 28046 Madrid, Spain (e-mail: joser.arribas@salud.madrid.org).

There is a close association between shortened telomere length in peripheral blood mononuclear cells and diseases of aging, including cardiovascular diseases, dementia, and cancer.[6]

Interestingly, multiple studies have reported shorter telomeres in-HIV-infected patients compared with HIV negative controls.[7–12] In HIV-infected patients, telomere attrition could be caused by inhibition of human telomerase by antiretroviral drugs, more specifically nucleos(t)ide reverse transcriptase inhibitors [N(t)RTIs].[13–15]

Two recent studies have reported that tenofovir (TFV) at therapeutic concentrations is a potent inhibitor of telomerase activity[16,17] causing telomere shortening in vitro. Of the currently recommended N(t)RTIs, TFV is a more potent inhibitor of telomerase than abacavir, lamivudine, or emtricitabine. In contrast, certain protease inhibitors (PIs) such as saquinavir can upregulate telomerase activity in vitro.[18] Although the inhibition of telomerase caused by N(t)RTIs has been repeatedly demonstrated in vitro, there are very limited data about the in vivo impact of different antiretroviral treatment (ART) regimens on telomere shortening. Indeed, to the best of our knowledge, there is no study that has explored the impact of TFV containing regimens on telomere length of HIV-infected patients receiving ART. This is a relevant issue because TFV administered as tenofovir difumarate (TDF) or tenofovir alafenamide is recommended as a preferred treatment option for initial treatment of HIV infection in all expert guidelines.

To try to determine the impact of TFV on telomere length of HIV-infected patients receiving ART, we have compared telomere length in a cohort of virologically suppressed, HIV-infected patients who were receiving antiretroviral regimens including and not including TDF. Our research hypothesis was that exposure to TDF would be associated with shorter telomere lengths.

PATIENTS, MATERIAL AND METHODS

Study Design and Population

A total of 103 HIV-infected patients exposed to treatment with TDF ("TDF exposed") and 97 HIV-infected patients who had never received TDF ("Non-TDF exposed"), aged >18 years old were included in the study. All patients were recruited from Hospital Universitario La Paz (Madrid, Spain) between March 2014 and March 2015. Main inclusion criteria included the following: HIV antibody positive, stable ART (defined as ART without changes in regimen for at least 12 months) and plasma HIV RNA of less than 50 RNA copies per milliliter for at least 1 year before recruitment. We offered participation in the study to all the patients who met inclusion criteria in our database.

Exclusion criteria were detectable viral load in the last 3 months before the inclusion (a unique viral load above 50 but below <200 RNA copies per milliliter was allowed during the 3 months before recruitment), current or previous treatment with chemotherapy or biologic treatments, acute infection with systemic repercussion during the 3 weeks before the inclusion, former or active alcoholism, pregnancy and type 2 HIV infection. Relevant demographics, parental age at birth of the participant, clinical,and behavioral data were also collected.

Variables

Patient's information regarding age, sex, race, chronic hepatitis C status, and HIV-related variables (Nadir CD4 count, transmission route, and AIDS stage) was collected retrospectively from clinical records. Researchers interviewed participants to self-report about parental age at birth, financial income, educational level, lifelong use tobacco, alcohol, and nonprescription drugs. Income <12,000 €/yr represented the median income cut-off in the Spanish region where our hospital is located.[19]

For cigarette smoking, data were recorded on a yes (active or former)/no basis, number of cigarettes per day and years. Given that the effect of tobacco over the telomere length would be cumulative and potentially irreversible,[20] cumulative exposure in "pack-years" was calculated as (cigarettes/d × years smoking)/20. Cumulative exposure to alcohol in "gr-years" was calculated as grams of alcohol/d × years drinking. Alcoholism was defined as daily alcohol consumption >70 gr/d in men and >40 gr/d in women.

Ethics Statement

The study was approved by the Ethics Committees of Hospital Universitario La Paz (Madrid, Spain). Written informed consent was obtained from all patients.

Sample Preparation: DNA Extraction

Genomic DNA was extracted from 1 mL of whole blood using MagPurix Blood DNA Extraction Kit 1200 according to manufacturer's instruction (Zinexts Life Science Corp., New Taipei City, Taiwan). DNA concentration was quantified using Qubit dsDNA BR assay kit (Life Technologies, Carlsbad, CA).

Telomere Length Determination by Quantitative Real-Time PCR

Relative telomere length was determined by monochrome quantitative multiplex polymerase chain reaction (PCR) assay[21] with minor modifications. Briefly, PCR reactions were performed on a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA) in a final volume of 20 μL containing 20 ng of genomic DNA, 1X PowerUp SYBR Green Master Mix (Applied Biosystem, Foster City, CA), and 900 nM final concentration of each telomere primers (telg, 5′-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3′ and telc, 5′-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3′) and albumin primers (albu, 5′-CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG-3′ and albd, 5′-GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT-3′). The thermal cycling profile was stage 1: 2 minutes at 50°C and 10 minutes at 95°C; stage 2: 2 cycles of 15 seconds at 94°C and 1.30 minutes at 49°C; and stage 3: 32 cycles of

15 seconds at 94°C, 15 seconds at 62°C, 15 seconds at 74°C with signal acquisition, 15 seconds at 84°C, and 15 seconds at 88°C with signal acquisition. A standard curve was prepared with genomic DNA from an HIV-negative patient by serial dilution and was included in each run in duplicate to assess amplification efficiency and linearity. All samples were run in triplicate and those with a SD of the threshold cycle (Ct) greater than 0.20 were reanalyzed.

Statistical Analysis

Characteristics of the sample were described using percentages for categorical variables, and mean (SD) or median (interquartile range) for continuous variables with normal or nonnormal distribution, respectively. χ^2, Student's t and Kruskal–Wallis were used accordingly for group comparisons.

Generalized linear models with log link function were fitted to evaluate the association of independent factors with telomere length. Variables independently associated with telomere length were evaluated using a backward stepwise procedure until all variables in the model had $P < 0.10$. Models were fitted separately for the full sample and the TDF exposed and nonexposed. Evaluated factors included: current age and sex, paternal and maternal age at birth, race, income, education, alcohol and tobacco consumption, use of injected drugs, HIV transmission route, hepatitis C virus coinfection and previous treatment with interferon, level of C-reactive protein, time with HIV infection, time on ART, time on nucleoside reverse transcriptase inhibitors (NRTI) and time on TDF (only for the group exposed to TDF), treatment with PIs, CD4 cell count nadir, and AIDS stage.

The specific effects of exposure to TDF (Yes/No), the time on NRTI (per 5 years) and for the group exposed to TDF, the total time on TDF (<5; 5–10; and >10 years) on telomere length were evaluated using an estimative approach, adjusting for age and evaluating the confounding introduced by the rest of independent variables. Finally, as an exploratory analysis, the association of telomere length and history of receiving PIs and time on PIs was evaluated with a similar approach.

Wald test was used to derive *P*-values. Analyses were conducted using STATA (V.13.0 MP; Stata Corporation, College Station, TX).

RESULTS

Characteristics of Study Participants

The characteristics of the participants exposed and nonexposed to TDF are listed in Table 1. Patients were on average predominantly men. There were no differences in sex, race, income, or educational level. TDF treated patients had a significantly higher consumption of alcohol, (58.3% vs 33%, $P = 0.001$), whereas we did not find differences in smoking habit or history of intravenous drug use.

HIV had been acquired mainly by sexual contact in both groups. More than 80% in each group had known their HIV infection for more than 10 years. Duration of known HIV infection was significantly longer in patients exposed to TDF. Patients nonexposed to TDF had been receiving ART for more than 10 years less frequently than patients exposed to TDF, but this difference did not reach statistical significance. All participants had ever received a regimen that contained an N(t)RTI, with longer duration of N(t)RTI exposure in the TDF exposed group, where more than 70% of the patients had received an N(t)RTI containing regimen for more than 10 years. Time of virological suppression was similar in both groups. Patients never exposed to TDF were more frequently receiving triple therapy, whereas patients exposed to TDF had more frequent use of boosted PI monotherapy. Current PI treatment and time on a PI regimen were similar in both groups. There were no differences in current CD4 count or nadir CD4 count.

Univariate Analyses of the Association Between Possible Predictors and Leukocyte Telomere Length

Younger age (<45 vs ≥50 years) and race different than white were significantly associated with longer telomere length among all participants (Fig. 1). No associations with sex, parental age at birth, educational level, or income were seen overall. Cumulative exposure to tobacco was associated with shorter telomere length, whereas history of intravenous drug abuse and cumulative alcohol consumption were not associated.

Longer time of known HIV infection was associated with shorter telomere length. Compared with patients who have known their HIV infection for less than 10 years, patients with known HIV infection for 10–20 and ≥20 years had telomeres that were 18% and 15% shorter ($P < 0.001$ for both).

Longer time on ART and longer time receiving N(t)RTI infection were associated with shorter telomere length. Exposure to TDF was not associated with telomere length, but among the exposed, those with longer exposure had longer telomeres, an effect of borderline statistical significance ($P = 0.060$). Also, those treated with PIs had shorter telomeres but only in the group not treated with TDF. Lower nadir CD4 cell counts, CD4 count, and AIDS stage were not associated with shorter telomere length (Table 1, Supplemental Digital Content, http://links.lww.com/QAI/B6).

Multivariate Analyses of the Association Between Possible Predictors and Leukocyte Telomere Length

Table 2 shows the results from the multivariate analysis overall and separately for the group exposed and nonexposed to TDF. Significant predictors of shorter telomere length, overall and in patients nonexposed to TDF, in a multivariate linear regression model included older age ($P = 0.008$), paternal age at birth ($P = 0.038$), and white race ($P = 0.048$). In addition, longer time of known HIV infection was associated with shorter telomere length (10–20 and ≥20 years compared with <10 years, $P = 0.003$ and $P = 0.056$, respectively).

TABLE 1. Participant Characteristics

	Total, n (%)	Non-TDF Group, n (%)	TDF Group, n (%)	*P*
N	200 (100)	97 (48.5)	103 (51.5)	
Sex				
Men	144 (72)	74 (76.3)	70 (68.0)	0.190
Women	56 (28)	23 (23.7)	33 (32)	
Age, median (IQR), yrs	49 (45–54.5)	49 (45–55)	49 (46–54)	0.993
<45	42 (21.0)	24 (24.7)	18 (17.5)	0.279
45–50	65 (32.5)	27 (27.8)	38 (36.9)	
≥50	93 (62.5)	46 (47.4)	47 (45.6)	
Paternal age at birth, mean (SD)	32.1 (6.7)	32.2 (7.4)	32.0 (6.1)	0.898
Maternal age at birth, mean (SD)	29.5 (6.1)	29.7 (6.4)	29.3 (0.56)	0.710
Race				
White	189 (94.5)	89 (91.8)	100 (97.1)	0.098
Other	11 (5.5)	8 (8.2)	3 (2.9)	
Income				
Lower (≤12,000 €/yr)	95 (47.5)	45 (46.4)	50 (48.5)	0.761
Higher (>12,000 €/yr)	105 (52.5)	52 (53.6)	53 (41.5)	
Education				
Primary	86 (43.0)	41 (42.3)	45 (43.7)	0.323
Secondary	61 (30.5)	26 (26.8)	35 (34.0)	
University	53 (26.5)	30 (30.9)	23 (22.3)	
Alcohol	91 (45.5)	32 (33)	59 (58.3)	0.001
Years, median (IQR)	29 (20–35)	28 (19–35)	30.5 (23–36)	0.172
Alcohol gr/wk, median (IQR)	45 (20–120)	50 (30–140)	40 (10–100)	0.104
Smoking	106 (53)	47 (48.5)	59 (57.3)	0.211
Years smoking, median (IQR)	31 (24–37)	33 (27–38)	30 (21–37)	0.264
Cigarettes per day, median (IQR)	15 (10–20)	15 (8–20)	15 (10–20)	0.818
Ever IDU	61 (30.5)	24 (24.7)	37 (35.9)	0.086
HIV transmission route				
Sexual	124 (62.0)	65 (67.0)	59 (57.3)	0.167
Parenteral	70 (35.0)	28 (28.9)	42 (40.8)	
Unknown	6 (3.0)	4 (4.1)	2 (1.9)	
HCV coinfection	40 (20.0)	14 (14.4)	26 (25.2)	0.097
Previous interferon treatment	40 (20.0)	16 (16.5)	24 (23.3)	0.229
Time with HIV infection, yrs, median (IQR)	18.48 (14.18–22.5)	16.9 (11.98–21.94)	19.39 (15.72–23.59)	0.007
<10	23 (11.5)	15 (15.5)	8 (7.8)	0.070
10–20	99 (49.5)	51 (51.6)	48 (46.6)	
≥20	78 (39.0)	31 (32.0)	47 (45.6)	
Time on ART, yrs, median (IQR)	14.92 (10.28–17.92)	14.34 (10.02–17.12)	15.0 (11.08–18.52)	0.09
<10	40 (20.0)	22 (22.7)	18 (17.5)	0.206
10–20	138 (69.0)	68 (70.1)	70 (68.0)	
≥20	22 (11.0)	7 (7.2)	15 (14.6)	
Time on NRTI, yrs, median (IQR)	11.95 (9.01–16.16)	11.07 (8.21–15.89)	12.68 (9.51–16.38)	0.2
<5	23 (11.5)	14 (14.4)	9 (8.7)	0.495
5–10	42 (21.0)	22 (22.7)	20 (19.4)	
10–15	71 (35.5)	31 (32.0)	40 (38.8)	
≥15	64 (32.0)	30 (30.9)	34 (33.0)	
Time on TDF, yrs, median (IQR)	—	—	8.48 (3.88–10.37)	—
<5	—	—	31 (30.1)	—
5–10	—	—	41 (39.8)	
≥10	—	—	31 (30.1)	
Time suppressed, (yrs) median (IQR)	6.79 (4.56–7.68)	6.89 (3.90–7.72)	6.70 (5.57–7.53)	0.99
Current ART regimen				
Triple therapy	128 (64.0)	69 (71.3)	59 (57.28)	<0.001
Boosted PI monotherapy	65 (32.5)	21 (21.25)	44 (42.7)	
NRTI-sparing regimen	7 (3.5)	7 (7.22)	—	

(*continued on next page*)

TABLE 1. (*Continued*) Participant Characteristics

	Total, n (%)	Non-TDF Group, n (%)	TDF Group, n (%)	*P*
Current NRTI back-bone	128 (64)	69 (71.3)	59 (57.28)	0.041
TDF/FTC	57 (44.53)	—	57 (96.61)	<0.001
ABC/3TC	65 (50.78)	64 (92.7)	1 (1.69)	
Other combinations	6 (4.68)	5 (7.25)	1 (1.69)	
Current boosted PI	100 (50.0)	50 (51.45)	50 (50.0)	0.671
Time on boosted PI	7.87 (4.82–11.0)	8.92 (5.43–11.0)	6.07 (4.20–11.12)	0.18
Ever exposed to PIs as part of ART	161 (80.5)	77 (79.4)	84 (81.6)	0.698
Time on PIs, yrs, median (IQR)	7.9 (4.8–11.0)	8.9 (5.4–11.0)	7.0 (4.2–11.1)	0.1802
<5	41 (25.5)	18 (23.4)	23 (27.4)	0.232
5–10	62 (38.5)	27 (35.1)	35 (41.7)	
≥10–15	31 (19.3)	20 (26.0)	11 (13.1)	
≥15	27 (16.8)	12 (15.6)	15 (17.9)	
CD4 count, cells/μL, median (IQR)	776 (551–1037)	801 (575–1080)	733 (519–1005)	0.21
Nadir CD4 count, cells/μL, median (IQR)	186 (92–276)	193 (93–289)	179 (87–246)	0.22
<100	52 (26)	24 (24.7)	28 (27.2)	0.278
100–200	81 (40.1)	36 (37.1)	45 (43.7)	
≥200	58 (29)	34 (35.1)	24 (23.3)	
Unknown	9 (4.5)	3 (3.1)	6 (5.8)	
Previous AIDS stage	106 (53.0)	51 (52.6)	55 (53.4)	0.907
Comorbidities				
Chronic kidney failure	8 (4.0)	6 (6.2)	2 (1.9)	0.126
High blood pressure	37 (18.5)	20 (20.6)	17 (16.5)	0.454
Diabetes mellitus	28 (14.0)	15 (15.5)	13 (12.6)	0.563
Treatment with statins	61 (30.5)	35 (36.1)	26 (25.2)	0.096
Blood inflammation biomarkers				
Reactive C protein, mg/L, median (IQR)	1.10 (0.37–3.79)	1.25 (0.42–4.46)	0.885 (0.29–2.99)	0.105
D-Dimer, ng/mL, median (IQR)	224 (<170–321)	230 (<170–321)	208 (171–319)	0.87
Fibrinogen, mg/dL, median (IQR)	330.5 (290–386.5)	332 (294–380)	329 (289–389)	0.92
Telomeres length (PCR), median, mean (IQR)	0.760, 0.782 (0.668–0.861)	0.749, 0.797 (0.685–0.861)	0.772, 0.768 (0.664–0.863)	0.962

IDU, injection drug user; IQR, interquartile range.

In the group nonexposed to TDF, shorter telomere length was associated with white race ($P = 0.016$) and longer time with HIV-infection (10–20 and ≥20 years compared with <10 years, $P = 0.048$ and $P = 0.067$, respectively).

In the group exposed to TDF, longer telomere length was associated with high educational level, lower income, and total time on ART, but not with other predictors, with the association with age in the limit of statistical significance.

Impact of Treatment Containing TDF on Telomere Length

Patients exposed to TDF had telomeres that were 4% shorter than those of patients who have never received TDF, but this difference did not reach statistical significance [exp(b) = 0.96; 95% confidence interval (CI): 0.90 to 1.03, $P = 0.238$], nor any other independent variable was identified as a confounder of this effect.

In the group of patients exposed to TDF, in the crude analysis, compared with those with less than 5 years of exposure, those with 5–10 years had 8.7% longer telomere length [exp(b) = 1.087; 95% CI: 0.997 to 1.185, $P = 0.060$] and those with over 10 years had 6.4% longer telomere length [exp(b) = 1.064; 95% CI: 0.970 to 1.167, $P = 0.189$], although the global significance of this association was far from statistical significance ($P = 0.163$), no confounders were identified.

Impact of Time Receiving N(t)RTIs on Telomere Length

Telomere length decreased with time on N(t)RTI, with 4% attrition for every 5 year of treatment with N(t) RTIs [exp(b) = 0.96; 95% CI: 0.93 to 0.99, $P = 0.01$]. However, after adjusting for age, the relationship between shorter telomere length and longer time on NRTI decreased to 3% and did not reach statistical significance (95% CI: 0.95 to 1.00, $P = 0.076$).

Impact of Time Receiving PIs on Telomere Length

At the crude level, in patients with previous exposure to PIs, telomere length was 7.6% shorter than in those not previously exposed [exp(b) = 0.924; CI 95%: 0.857 to 0.996; $P = 0.039$]. However, further adjustment by age and

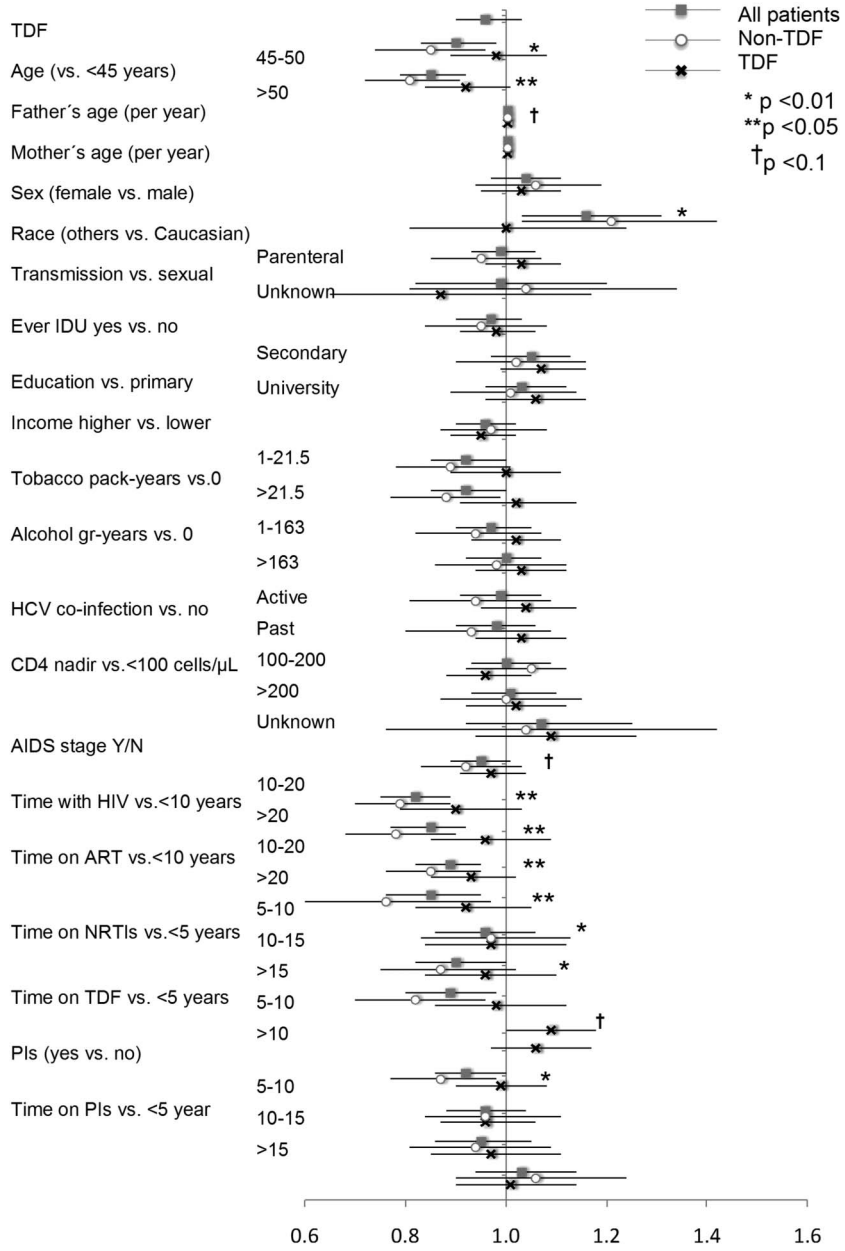

FIGURE 1. Univariate analyses of the association between possible predictors and leukocyte telomere length.

total time on ART made this association disappear [exp(b) = 0.953; CI 95%: 0.884 to 1.027; $P = 0.204$]. In the group of patients exposed to PI, no association was evident between length of exposure and telomere length, and no confounders were identified.

DISCUSSION

In our study, we have shown that ART including TDF does not seem to have an intrinsic negative impact on telomere length in peripheral blood of HIV-infected patients with virological suppression. Compared with patients who have never received TDF, patients who have received TDF for a prolonged period of time—70% for more than 5 years—did not have shorter telomeres in a multivariate analysis looking specifically at the impact of TDF on telomere length.

Ours is the first study that has looked specifically for an in vivo effect of TDF on telomere length in HIV-infected patients. For this reason, we ought to compare a group of patients who have received long-term treatment with TDF with a group of patients who have never been treated with TDF. Both groups were highly comparable in terms of factors that in previous studies have been associated with telomere length: sex distribution, age, income, and smoking status.[6] Besides, it has been shown that HIV by itself can down-regulate telomerase activity.[22–24] Importantly, all patients in both groups have prolonged virological suppression. Consequently, our results are not affected by differences between

TABLE 2. Multivariate Analyses of the Association Between Possible Predictors and Leukocyte Telomere Length

	All		Non-TDF Exposed		TDF Exposed	
Variable	**Exp(coef.) CI (95%)**	***P***	**Exp(coef.) CI (95%)**	***P***	**Exp(coef.) CI (95%)**	***P***
Age (Ref. <45 yrs)						
≥45/50	0.95 (0.87 to 1.03)	0.216	0.91 (0.79 to 1.04)	0.169	0.99 (0.90 to 1.10)	0.908
≥50	0.90 (0.83 to 0.97)	0.008	0.88 (0.77 to 1.01)	0.065	0.92 (0.83 to 1.01)	0.074
Father's age at birth (per yr)	1.005 (1.000 to 1.009)	0.038	1.006 (0.999 to 1.013)	0.080	—	
Race (Ref. Caucasic)						
Other	1.13 (1.00 to 1.28)	0.048	1.22 (1.04 to 1.43)	0.016	—	
Education (Ref. Primary)						
Secondary	—		—		1.12 (1.03 to 1.22)	0.006
University	—		—		1.10 (1.00 to 1.21)	0.044
Income (Ref. Low)						
High					0.92 (0.86 to 0.99)	0.031
Time with HIV infection (Ref. <10 yrs)						
≥10–20	0.87 (0.79 to 0.95)	0.003	0.86 (0.74 to 1.00)	0.048	—	
≥20	0.91 (0.82 to 1.00)	0.056	0.86 (0.74 to 1.01)	0.067	—	
Time on ART (Ref. <10 yrs)						
≥10–20	—		—		0.89 (0.82 to 0.98)	0.017
≥20	—		—		0.91 (0.80 to 1.03)	0.120

groups in virological control. Finally, both groups were comparable with regard to total time receiving ART or time receiving N(t)RTIs, 2 factors that theoretically could affect telomere length.[16,17] It is important to highlight that our participants treated with TDF had a longer duration of N(t) RTI exposure and despite this fact, TDF exposure was not associated with shorter telomeres.

There are very few previous studies that have explored in vivo the impact of different types of ART regimens on telomere length and none that have specifically focused on the impact of TDF. Leeansyah et al[17] in a cross-sectional study with a small sample of just 53 patients found in a univariate analysis that duration of N(t)RTI-containing ART was inversely associated with telomere length and that there was no association with telomerase activity. However, in a multivariate analysis, duration of N(t)RTI-containing ART was no longer significantly related to telomere length. In a substudy of the MONET clinical trial, comparing darunavir/ritonavir monotherapy versus darunavir/ritonavir and 2 N(t)RTIs for maintenance of virological suppression, there was no significant association between telomere length and the duration of previous N(t)RTI treatment.[25] Besides, in MONET there were no significant differences between the 2 arms after 3 years of follow-up in telomerase activity or mean change per year of telomere length. Finally, in a cohort of 229 HIV-infected patients, Zanet et al[7] found no evidence of a relationship between telomere length and antiretroviral therapy exposure, or current type of antiretroviral therapy, although in this study the prevalence of treatment with TDF was not reported.

In our multivariate analysis, longer time of HIV infection was associated with shorter telomere length. Compared with patients with less than a decade of known HIV infection, patients with longer durations of known HIV infection had telomeres that were 9%–13% shorter. Time with HIV infection had substantial colinearity with time receiving ART and time receiving NRTIs. When we included these 3 variables in our model, the effect of total time on ART and time receiving NRTI on telomere shortening was explained by total time with known HIV infection. Our study adds further evidence that HIV by itself or by causing persistent inflammation, immune activation, or immune senescence seem to be the predominant factors causing telomere shortening and not the use of specific antiretrovirals such as TDF.[7–9,11,12,26]

The other variables associated with telomere shortening were as expected, older age[20] and parental age at birth.[27] In our study white race was also associated with shorter telomere length. This finding has to be considered with caution because the impact of race on telomere length is controversial with conflicting findings depending on the study and the sample analyzed.[28]

Our study is limited by its cross-sectional nature that leads to unmeasured bias in the distribution of different ART regimens. However, we think that our results are inconsistent with a large effect of TDF on telomere shortening at least in peripheral blood in HIV-infected patients. Despite the fact that TDF is the strongest inhibitor of telomerase activity in vitro, this effect seems to be compensated by an unknown mechanism in vivo. One possible explanation is that because HIV-Tat protein by itself can downregulate telomerase expression and activity,[24] the negative effect of TDF seen in vitro is compensated by its antiviral activity in vivo. If the inhibition of telomerase caused directly by HIV is substantially higher than the inhibition caused by TDF, then the net effect of TDF on telomere shortening could be positive and similar to other antiretrovirals. We explored also if treatment with PIs could antagonize the negative effect of TDF because an in vitro study has shown that saquinavir can upregulate telomerase activity.[18] However, results of our analysis do not support this hypothesis. Additional mechanisms by which telomerase inhibition may lead to abnormal

aging in the absence of shorter telomeres include DNA damage induced by deprotection of telomeres that may finally result in premature senescence.[29]

Another limitation of our study is that we did not determine telomere length on $CD4^+$ or $CD8^+$ T cells, specific subsets of T cells or non–T-cell population or in high replicating tissues. It remains possible that the effect of TDF on the telomerase of these cell subsets could be different from its overall effect on whole blood. Beside, TDF levels could be different depending on the body compartment, and its impact in the different tissues should be studied. In addition, time of HIV infection was no precisely measured in our study because this variable was calculated from the time of diagnosis and not from the actual time of seroconversion. Finally, because ours was a retrospective study using medical records, and for certain variables such as complete antiretroviral history, not all the data were available.

Our results do not completely rule out that there are in vivo differences among antiretrovirals in its ability to produce telomere shortening in vivo. We recognize that a better control group would be HIV-infected patients never exposed to nucleosides, because other nucleosides such as abacavir have an impact on telomerase activity.[16,17] The issue of a different impact of various types of ART on telomere length would not be definitively answered if this endpoint were not measured in a randomized clinical trial comparing different ART regimens. In particular, it would be very interesting to compare in a prospective randomized study the impact on telomere length in different cells and tissues of N(t) RTI-containing and N(t)RTI-sparing regimens in naive patients who start ART.

In summary, we have found that despite its confirmed ability to inhibit telomerase in vitro, ART including TDF does not seem to lead to telomere shortening in the peripheral blood of HIV-infected patients.

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Publicación 3

IMPACT OF NUCLEOS(T)IDE REVERSE TRANSCRIPTASE INHIBITORS ON BLOOD TELOMERE LENGTH CHANGES IN A PROSPECTIVE COHORT OF AVIREMIC HIV-INFECTED ADULTS

En línea con las publicaciones anteriores, en este trabajo el principal objetivo era el estudio del impacto de los N(t)RTIs, especialmente TDF, en la LT. En el estudio previo de corte transversal no se han detectado diferencias significativas en la LT entre el grupo expuesto a TDF y el grupo no expuesto. Tras dos años de seguimiento de los pacientes de la cohorte con replicación viral suprimida del Hospital Universitario La Paz, se ha realizado un estudio longitudinal para determinar si se producen cambios en la LT en sangre. Para ello, al igual que en el estudio transversal, se ha comparado la LT de los pacientes expuestos y no expuestos a TDF. Es importante destacar que existen diferencias en el criterio de inclusión de los pacientes expuestos a TDF entre ambos estudios. En el estudio de corte transversal los pacientes incluidos en el grupo con exposición a TDF habían recibido en algún momento de su TAR una pauta que incluía TDF, sin embargo al momento del inicio del estudio sólo el 55% de los pacientes estaban recibiendo TDF. Por el contrario, en el estudio longitudinal se incluyeron en el grupo con exposición a TDF sólo a aquellos pacientes que estaban recibiendo TDF como parte del TAR al momento del inicio del estudio. Los resultados obtenidos en este estudio longitudinal indican que en los pacientes VIH+ avirémicos tras dos años de seguimiento las pautas de TAR que incluyen TDF o ABC se asocian a una menor ganancia de la LT.

Mi contribución personal en la parte experimental de este trabajo consistió en la recogida y procesamiento de las muestras de seguimiento de los pacientes de la cohorte y la determinación de la LT en sangre. En cuanto a la elaboración del manuscrito he participado en el análisis de los resultados obtenidos, el diseño de figuras y tablas y la escritura del artículo.

The Journal of Infectious Diseases

MAJOR ARTICLE

Impact of Nucleos(t)ide Reverse Transcriptase Inhibitors on Blood Telomere Length Changes in a Prospective Cohort of Aviremic HIV–Infected Adults

Rocio Montejano,[1,a] Natalia Stella-Ascariz,[1,a] Susana Monge,[1] José I. Bernardino,[1] Ignacio Pérez-Valero,[1] Maria Luisa Montes,[1] Eulalia Valencia,[1] Luz Martín-Carbonero,[1] Victoria Moreno,[1] Juan González-Garcia,[1] Javier Rodriguez-Centeno,[1] Berta Rodes,[1] Andres Esteban Cantos,[1] Belen Alejos,[2] Rosa de Miguel,[1] Francisco Arnalich,[1] Rosario Perona,[3] and José R. Arribas[1]

[1]Hospital Universitario La Paz–IdiPAZ, [2]Instituto de Salud Carlos III, and [3]Instituto de Investigaciones Biomédicas CSIC/UAM, IdiPAZ, Biomarkers and New Therapies and CIBER de Enfermedades Raras, Madrid, Spain

(See the Editorial Commentary by Côté and Hsieh on pages 1521–2 and Major Article by Stella-Ascariz et al on pages 1523–30.)

Background. Tenofovir is a potent inhibitor of human telomerase. The clinical relevance of this inhibition is unknown.

Methods. A prospective cohort of human immunodeficiency virus (HIV)–infected participants with suppressed virological replication was recruited to compare whole-blood telomere length (measured by quantitative multiplex polymerase chain reaction analysis) in participants with current exposure to tenofovir disoproxil fumarate (TDF) to that in participants never exposed to TDF.

Results. A total of 172 participants were included: 67 were in the TDF group, and 105 were in the non-TDF group (75 were receiving 2 nucleosides [of whom 69 were receiving abacavir], 25 were receiving a nucleos[t]ide reverse transcriptase inhibitor [N{t}RTI]–sparing regimen, and 5 were receiving lamivudine as the only nucleoside). After 2 years, the mean blood telomere length increased significantly in the whole cohort. The TDF group had significantly smaller gains in telomere length than the non-TDF group. In the analysis restricted to participants receiving N(t)RTIs, TDF exposure was not associated with an independent negative effect. In the non-TDF group, participants treated with 2 nucleosides also had significantly smaller gains in telomere length than those receiving N(t)RTI-sparing regimens or lamivudine as the only nucleoside.

Discussion. In HIV-infected adults with prolonged virological suppression, treatment with TDF or abacavir was associated with smaller gains in blood telomere length after 2 years of follow-up.

Keywords. HIV infection; antiretroviral therapy; tenofovir; suppressed; abacavir; telomerase; telomere length.

Compared with uninfected adults, human immunodeficiency virus (HIV)–infected individuals have shorter blood telomere length (TL) [1, 2]. After HIV seroconversion, there is a rapid and substantial decrease in blood TL [3, 4]. Multiple mechanisms can contribute to TL attrition during HIV infection: inhibition of telomerase by HIV proteins such as HIV-Tat [5], chronic antigenic stimulation leading to immunosenescence (in which well-differentiated T cells with shorter telomeres predominate) [6], and direct inhibition of telomerase caused by nucleos(t)ide reverse transcriptase inhibitors (N[t]RTIs). In vitro studies have shown that the N(t)RTIs tenofovir and abacavir inhibit human telomerase, with tenofovir being the most potent inhibitor [7–9]. The clinical relevance of this in vitro finding is unknown.

To evaluate the impact of tenofovir disoproxil fumarate or other N(t)RTIs upon TL, we performed the first prospective study of blood TL changes in HIV-infected individuals in whom virological replication was suppressed during treatment with tenofovir disoproxil fumarate–containing or tenofovir disoproxil fumarate–sparing antiretroviral therapy (ART). Our research hypothesis, in line with results of in vitro studies, was that continuous exposure to tenofovir disoproxil fumarate would have a negative impact on blood TL changes.

PARTICIPANTS, MATERIALS, AND METHODS

Study Design and Population

A total of 67 HIV-infected participants currently receiving tenofovir disoproxil fumarate and 105 HIV-infected participants who had never received tenofovir disoproxil fumarate were included in this cohort. All participants were recruited from Hospital Universitario La Paz (Madrid, Spain) HIV unit between March 2014 and March 2015. Main inclusion criteria were as follows: age of >18 years, HIV antibody positive, stable ART regimen (no change in ART for at least 12 months), and serum HIV RNA load of <50 RNA copies/mL for at least 1 year prior to recruitment (a unique viral load of >50 but <200 RNA

Received 28 February 2018; editorial decision 30 April 2018; accepted 13 June 2018; published online June 15, 2018.

Presented in part: 16th European AIDS Conference, Milan, Italy, 25–27 October 2017. Abstract BPD2/9.

[a]R. M. and N. S.-A. contributed equally to this report.

Correspondence: J. R. Arribas, MD, Hospital La Paz, IdiPAZ, Edificio de Investigación, Despacho 3.3, Paseo de la Castellana 261, 28046, Madrid, Spain (joser.arribas@salud.madrid.org).

The Journal of Infectious Diseases® 2018;218:1531–40

DOI: 10.1093/infdis/jiy364

copies/mL was allowed up to 3 months before study entry). We offered participation in the study to all patients who met inclusion criteria in our database.

Exclusion criteria were as follows: current or previous treatment with chemotherapy or biologic agents, acute infection with systemic repercussion within the 3 weeks prior to study entry, former or active alcoholism (consumption of >70 g of alcohol per day for men and 40 g/day for women), pregnancy, and HIV infection. We obtained relevant demographic, clinical, and behavioral data and parental age at birth of the participant.

Follow-up and Variables

The study included 1 visit at study entry and 1 visit after 2 years for follow-up. Participants age, sex, race, chronic hepatitis C status (defined as a detectable hepatitis C virus RNA at study entry), and HIV-related variables (nadir $CD4^+$ T-cell count, transmission route, and AIDS stage), ART history, use of N(t)RTIs (at study entry and the follow-up visit), and use of tenofovir disoproxil fumarate at follow-up were collected from clinical records. Researchers interviewed participants about parental age at birth, financial income, education level, and use of tobacco, alcohol, and other recreational drugs (active vs former/never). No other specific intervention was performed, and participants entering the study were managed according to routine clinical practice. An income of €12 000/year (approximately $14 000/year) represented the mean income in the Spanish region where our hospital is located [10].

Laboratory Results

A blood specimen obtained after ≥8 hours of fasting was collected from all subjects at study entry and the follow-up visit at 96 weeks for real-time measurements of glucose level, creatinine level, lipid profile (including total, low-density lipoprotein, and high-density lipoprotein cholesterol levels and triglycerides level), and other biomarkers (high-sensitivity C-reactive protein [CRP] level, fibrinogen level, and D-dimer level). $CD4^+$ T-cell count and serum HIV RNA load were concomitantly measured as markers of HIV disease severity.

Ethics Statement

The study was approved by the Ethics Committee of Hospital Universitario La Paz (Madrid, Spain). Written informed consent was obtained from all participants.

TL Determination

Genomic DNA was isolated from whole-blood specimens, using the MagPurix Blood DNA Extraction Kit 1200 (Zinexts Life Science, New Tapei City, Taiwan) according to the manufacturer's instructions. Relative TL, expressed as the ratio of the telomere amplification product (T) to that of a single-copy gene (S), was determined by monochrome quantitative multiplex polymerase chain reaction (PCR) assay as described in our previous study [11]. A standard curve with genomic DNA from a pool of 3 HIV-infected participants was prepared by serial dilutions and included in each run, together with a reference sample and negative control. Study entry and follow-up samples were assayed together on the same PCR plate. All samples were run in triplicate, and those with a coefficient of variation of >15% were reanalyzed. TL for a given participant was calculated as the mean of their repeated TL measurements. The intraassay coefficients of variation for the T/S ratio, T–cycle threshold (Ct), and S-Ct were 5.74%, 0.45%, and 0.33%, respectively, whereas the interassay coefficients of variation were 7.81%, 0.78%, and 1.35%, respectively.

Statistical Analysis

Sample characteristics were described using absolute and relative frequencies for categorical and means ± standard deviations for continuous variables. Differences by treatment group were analyzed using χ^2 or Student *t* tests as appropriate.

Intention-to-continue-treatment (ITCT) analysis, ignoring treatment changes, was performed for all treatment groups at study entry. An additional as-treated (AT) analysis was done for the subgroup of participants who received their original ART regimen throughout the study. Mean annual change in TL between study entry and follow-up was calculated and modeled by means of a linear regression analysis that adjusted for the study entry TL. Because follow-up times were not equal between patients, mean annual change in TL between baseline and follow-up was calculated and further modeled by means of a linear regression analysis that adjusted for the baseline TL.

In the main analysis, we compared participants receiving tenofovir disoproxil fumarate to those not receiving tenofovir disoproxil fumarate, to analyze the effect of tenofovir disoproxil fumarate on TL change. We did this for all participants and for the subgroup of participants who received N(t)RTIs, to try to differentiate the effect of tenofovir disoproxil fumarate as compared to other N(t)RTIs. We also compared participants receiving tenofovir disoproxil fumarate to participants receiving N(t)RTI-sparing regimens. Since 3 in vitro studies have shown that lamivudine or emtricitabine do not inhibit telomerase at therapeutic concentrations [7–9], ART regimens including lamivudine as the only N(t)RTI were classified as N(t)RTI sparing. Multivariable models were used to control for confounding due to measured independent factors. We estimated the most unbiased association between tenofovir disoproxil fumarate and TL change by using an estimative approach for model selection and thus retained in the model all variables that produced a change of ≥15% in the regression coefficient for the association between tenofovir and TL. Similarly, a secondary analysis was performed to compare participants receiving any N(t)RTIs or N(t)RTIs other than tenofovir disoproxil fumarate to participants not receiving N(t)RTIs. We did not adjust *P* values for multiple comparisons.

To complement our analysis, we created a predictive model including all variables to identify which variables could be

independent predictors of TL change during follow-up. We developed a saturated model by selecting variables showing a univariate association with a P value of < .20 and sequentially dropping variables until all showed a P value of < .05. Discarded variables were reevaluated and included again if they showed an association with a P value of < .10, so we could observe effects within the limit of statistical significance.

Model assumptions were verified by means of residuals diagnostics (data not shown). The Wald test was used to derive P values.

RESULTS

Characteristics of Study Participants

Characteristics at study entry of the participants with and those without tenofovir disoproxil fumarate exposure are listed in Table 1 and Supplementary Table 3. Participants were on average predominantly male, and there were no other statistically significant differences in sex, race, income, or education level. Tenofovir disoproxil fumarate–exposed participants reported significantly greater alcohol consumption at study entry. We did not find differences in smoking or history of recreational drug use.

HIV had been acquired mainly by sexual transmission in both groups. There were no statistically significant differences in time since HIV infection diagnosis (>16 years in both groups), duration of virological suppression before enrollment, or $CD4^+$ T-cell count at study entry.

At study entry, 71.4% of participants never exposed to tenofovir disoproxil fumarate were receiving triple-drug therapy based mainly on abacavir, and 28.5% were receiving an N(t)RTI-sparing regimen. For 5 participants, N(t)RTI-sparing regimens included lamivudine as the only nucleoside.

Blood TL at Study Entry and After 2 Years of Follow-up, by Regimen

Mean TL increased 0.042 (95% CI, .004–.079) in the whole cohort (P = .030). In the ITCT analysis, blood TL at study entry was not significantly different between participants receiving and those not receiving tenofovir disoproxil fumarate. Mean annual change in blood TL was also similar in both groups (0.025 in the non–tenofovir disoproxil fumarate group and 0.014 in the tenofovir disoproxil fumarate group). However, after 2 years, the intergroup difference in mean TL was statistically significant, with a longer TL in the non–tenofovir disoproxil fumarate group (Figure 1 and Supplementary Table 1*A*). The proportion of participants who had a TL increase at follow-up was also similar in both groups. These results did not change in the AT analysis (Supplementary Table 1*B*).

At study entry, we also found that participants receiving tenofovir disoproxil fumarate or any N(t)RTIs had significantly shorter TL than those in N(t)RTI-sparing regimens. At follow-up, participants receiving N(t)RTI-sparing regimens had significantly longer TL than participants receiving tenofovir disoproxil fumarate, N(t)RTIs, or NRTI-containing regimens (Figure 1 and Supplementary Table 1*A*).

Estimative Analysis of the Impact of Different ART Regimens on Blood TL Changes

Tenofovir Disoproxil Fumarate Regimens Versus Tenofovir Disoproxil Fumarate–Sparing Regimens

In the crude ITCT analysis, exposure to tenofovir disoproxil fumarate was associated with a trend toward a negative impact on TL. After 2 years, tenofovir disoproxil fumarate–exposed participants had gains in mean blood TL that were −0.0319 inferior (95% CI, −.0648–.010) to those for participants without non–tenofovir disoproxil fumarate exposure. After adjustment for alcohol consumption, the difference in favor of the non–tenofovir disoproxil fumarate group became statistically significant, with a mean difference in TL gain of −0.0391 (95% CI, −.0729 to −.0053).

In the AT population, we found a similar estimate of the negative effect of tenofovir disoproxil fumarate exposure on TL changes. In the crude analysis, exposure to tenofovir disoproxil fumarate was associated with a nonsignificant negative trend. Again, after adjustment for alcohol consumption, the difference increased and reached statistical significance (Figure 2 and Supplementary Table 2). When we restricted the analysis to participants receiving N(t)RTIs, we did not find a significant independent effect of tenofovir disoproxil fumarate on TL changes, regardless of the analysis.

Tenofovir Disoproxil Fumarate Regimens Versus N(t)RTI-Sparing Regimens

The difference in gains in TL in favor of participants receiving N(t)RTI-sparing regimens was −0.0722 (95% CI, −.1237 to −.0207). No confounders were identified. The AT analysis showed similar results, with a difference of −0.0707 (95% CI, −.1270 to −.0144).

N(t)RTI Regimens Versus N(t)RTI-Sparing Regimens

By ITCT analysis, exposure to N(t)RTIs had a negative impact on TL changes that was statistically significant. In the crude analysis, participants not exposed to N(t)RTIs at study entry had a significantly higher mean increase in blood TL as compared to participants without exposure to N(t)RTIs at study entry. In the crude analysis, the difference in favor of participants receiving N(t)RTI-sparing regimens was −0.0592 (95% CI, −.1011 to −.0172). Similar results were found in the AT population, with a statistically significant lower gain in TL in participants exposed to N(t)RTI at study entry (mean difference, −0.0593; 95% CI, −.1027 to −.0159). Further adjustment by time since HIV infection diagnosis increased the difference in TL in favor of participants receiving N(t)RTI-sparing regimens to −0.0689.

NRTI Regimens Versus N(t)RTI-Sparing Regimens

Finally, we also compared NRTI regimens and N(t)RTI-sparing regimens. By ITCT analysis, exposure to NRTIs had a negative impact on TL changes that was statistically

Table 1. Participant Characteristics, by Treatment Regimen

Characteristic	Non-TDF[a] (n = 105)	TDF[b] (n = 67)	*P*[c]	N(t)RTI[d] (n = 142)	NRTI[e] (n = 75)	N(t)RTI Sparing[f] (n = 30)
Age, y	49.7 ± 9.8	49.4 ± 7.5	.791	49.5 ± 8.8	49.7 ± 9.9	49.8 ± 9.8
Female sex	26 (24.8)	20 (29.9)	.462	38 (26.8)	18 (24.0)	8 (26.7)
Follow-up time, mo	24.8 ± 2.4	24.3 ± 1.9	.204	24.7 ± 2.3	25.1 ± 2.5	24.0 ± 1.7
Father's age at birth, y	32.5 ± 7.0	32.8 ± 5.5	.759	32.4 ± 6.6	32.0 ± 7.4	33.9 ± 5.9
Mother's age at birth, y	29.7 ± 6.2	30.2 ± 5.5	.559	29.7 ± 6.0	29.2 ± 6.3	30.9 ± 5.9
Ethnicity						
White	96 (91.4)	64 (95.5)	.304	131 (92.3)	67 (89.3)	29 (96.7)
Other	9 (8.6)	3 (4.5)		11 (7.8)	8 (10.7)	1 (3.3)
Education level						
Primary	42 (40.0)	24 (35.8)	.527	52 (35.2)	26 (34.7)	16 (53.3)
Secondary	32 (30.5)	26 (38.8)		49 (34.5)	23 (30.7)	9 (30.0)
University	31 (29.5)	17 (25.4)		43 (30.3)	26 (34.7)	5 (16.7)
Income level						
Low	49 (46.7)	27 (40.9)	.683	63 (44.4)	36 (48.0)	13 (43.3)
High	55 (52.4)	39 (59.1)		77 (54.2)	38 (50.7)	17 (56.7)
Unknown	1 (1.0)	1 (1.5)		2 (1.4)	1 (1.3)	0 (0.0)
Current tobacco use	50 (47.6)	36 (53.7)	.434	67 (47.2)	31 (41.3)	19 (63.3)
Current alcohol use	36 (34.3)	40 (59.7)	.002	62 (43.7)	22 (29.3)	14 (46.7)
HCV coinfection at study entry						
No	72 (68.6)	42 (62.7)	.712	98 (69.0)	56 (74.7)	16 (53.3)
Active	11 (10.0)	9 (13.4)		17 (12.0)	8 (10.7)	3 (10.0)
Comorbidity						
Chronic kidney disease	5 (4.8)	1 (1.5)	.254	5 (3.5)	4 (5.3)	1 (3.3)
Hypertension	23 (21.9)	12 (17.9)	.526	29 (20.4)	17 (22.7)	6 (20.0)
Diabetes mellitus	15 (14.3)	9 (13.4)	.875	20 (14.1)	11 (14.7)	4 (13.3)
Statin receipt	37 (35.2)	15 (22.4)	.074	41 (28.9)	26 (34.7)	11 (36.7)
Route of HIV transmission						
Sexual	70 (66.7)	45 (67.2)	.670	98 (69.0)	53 (70.7)	17 (56.7)
Parenteral	31 (29.5)	21 (31.3)		41(28.9)	20 (26.7)	11 (36.7)
Unknown	4 (3.8)	1 (1.5)		3 (2.1)	2 (2.7)	2 (6.7)
Previous AIDS	59 (56.2)	37 (55.2)	.901	77 (54.2)	40 (53.3)	19 (63.3)
Time since HIV infection diagnosis, y	16.3 ± 6.4	18.1 ± 6.0	.067	16.6 ± 6.4	15.2 ± 6.5	19.1 ± 5.4
Time with HIV load <50 copies/mL, y	6.7 ± 2.8	7.2 ± 1.9	.209	6.7 ± 2.3	6.3 ± 2.6	7.4 ± 3.3
N(t)RTI regimen at study entry						
Overall	75 (71.4)	67 (100)		142 (100)	75 (100)	5 (17.0)
ABC/3TC	69 (92.0)	…		69 (48.5)	69 (92.0)	…
TDF/FTC	…	64 (95.5)		64 (45.1)	…	…
AZT/3TC	3 (4.0)	…		3 (2.1)	3 (4.0)	…
TDF + 3TC	…	2 (2.99)		2 (1.4)	…	
3TC	…	…		…	…	5 (17.0)
ABC/3TC/AZT	1 (1.33)	…		1 (0.7)	1 (1.3)	…
ABC	1 (1.33)	…		1 (0.7)	1 (1.3)	…
3TC + ddI	1 (1.33)	…		1 (0.7)	1 (1.3)	…
3TC + d4T + TDF	…	1 (1.49)		1 (0.7)	…	…
N(t)RTI-sparing regimen						
Overall	30 (28.5)	…		…	…	30 (100)
PI monotherapy	22 (73.3)	…		…	…	22 (73.3)
PI + 3TC	3 (10.0)	…		…	…	3 (10.0)
LPV/r + ETV	1 (3.3)	…		…	…	1 (3.3)
LPV/r + RAL	1 (3.3)	…		…	…	1 (3.3)
ETV + RAL	1 (3.3)	…		…	…	1 (3.3)
3TC + ETV + RAL	1 (3.3)	…		…	…	1 (3.3)
3TC + ATV + RAL	1 (3.3)	…		…	…	1 (3.3)

Table 1. Continued

Characteristic	Non-TDF[a] (n = 105)	TDF[b] (n = 67)	*P*[c]	N(t)RTI[d] (n = 142)	NRTI[e] (n = 75)	N(t)RTI Sparing[f] (n = 30)
CD4+ T-cell count, cells/mL						
At study entry	846 ± 365	744 ± 340	.069	806 ± 349	862 ± 350	806 ± 401
At follow-up	824 ± 353	772 ± 313	.331	816 ± 341	860 ± 363	741 ± 319
Change	−13 ± 223	29 ± 164	.192	14 ± 196	0.42 ± 223	−44 ± 225
HIV RNA load <50 copies/mL at follow-up	104 (100)	67 (100)	…	142 (100)	75 (100)	29 (100)

Data are no. (%) of participants or mean (± standard deviation).

Abbreviations: ABC, abacavir; ATV, atazanavir; AZT, zidovudine; d4T, stavudine; ddI, didanosine; ETV, etravirine; FTC, emtricitabine; HCV, hepatitis C virus; HIV, human immunodeficiency virus; LPV/r, lopinavir/ritonavir; N(t)RTI, nucleos(t)ide reverse transcriptase inhibitor; PI, protease inhibitor; RAL, raltegravir; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine.

[a]Regimens not containing TDF.

[b]Regimens containing TDF.

[c]For comparison between the non-TDF and TDF groups.

[d]Regimens containing any N(t)RTI (except 3TC alone) or TDF.

[e]Regimens containing any nucleoside reverse transcriptase inhibitor (NRTI; ie, ABC, AZT, ddI, 3TC, or FTC) except 3TC alone.

[f]Regimens without N(t)RTIs or with 3TC only.

significant. In the crude analysis, participants not exposed to N(t)RTIs at study entry had a significantly higher mean increase in blood TL than participants with exposure to NRTIs at study entry. In the crude analysis, the difference in favor of participants receiving N(t)RTI-sparing regimens was −0.0513 (95% CI, −.0991–.0034). Similar results were found in the AT population. Further adjustment by time since HIV infection diagnosis increased the difference in TL in favor of participants receiving N(t)RTI-sparing regimens to −0.0682.

Predictive Model of Factors Associated to Annual Change in Blood TL

In the univariate analysis, older age was significantly associated with a lower mean TL gain among all participants. Female sex was associated with a trend toward a positive impact of borderline significance (Table 2 and Supplementary Figure 1). No associations with race, parental age at birth, education level, or income was observed. Regarding HIV-related factors, there was a trend toward an association between longer duration since HIV infection diagnosis and smaller increases in mean TL ($P = .051$). Exposure to N(t)RTIs at study entry was significantly

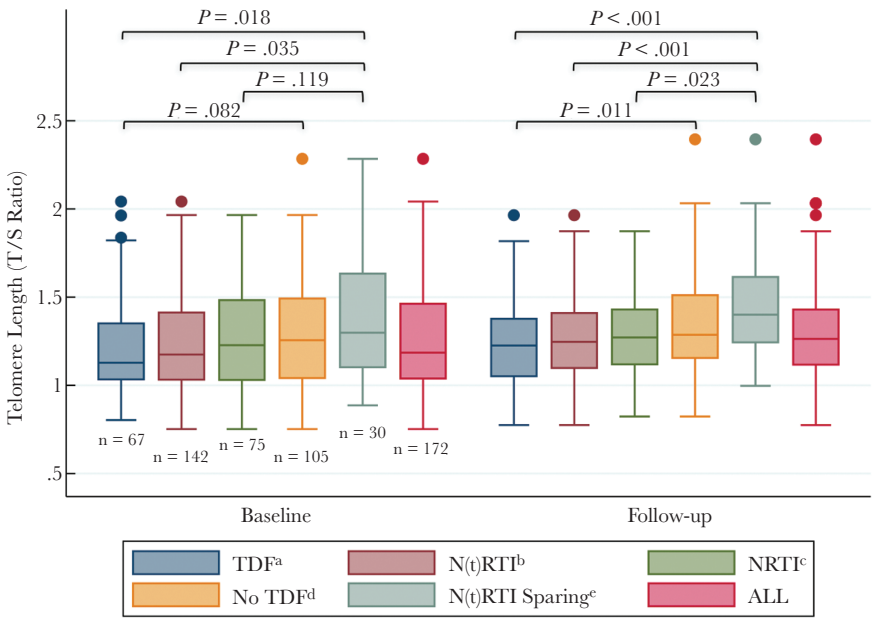

Figure 1. Intention to continue treatment analysis of blood telomere length at study entry and after 2 years of follow-up, by regimen. *P* values were determined by the Kruskal-Wallis test. N(t)RTI, nucleos(t)ide reverse transcriptase; TDF, tenofovir disoproxil fumarate; T/S ratio, ratio of the telomere amplification product to that of a single-copy gene; 3TC, lamivudine. [a]Regimens containing TDF. [b]Regimens containing any N(t)RTI (except 3TC alone) or TDF. [c]Regimens containing any nucleoside reverse transcriptase inhibitor (NRTI; ie, abacavir, atazanavir, didanosine, 3TC, or emtricitabine) except 3TC alone. [d]Regimens not containing TDF. [e]Regimens without N(t)RTIs or with 3TC only.

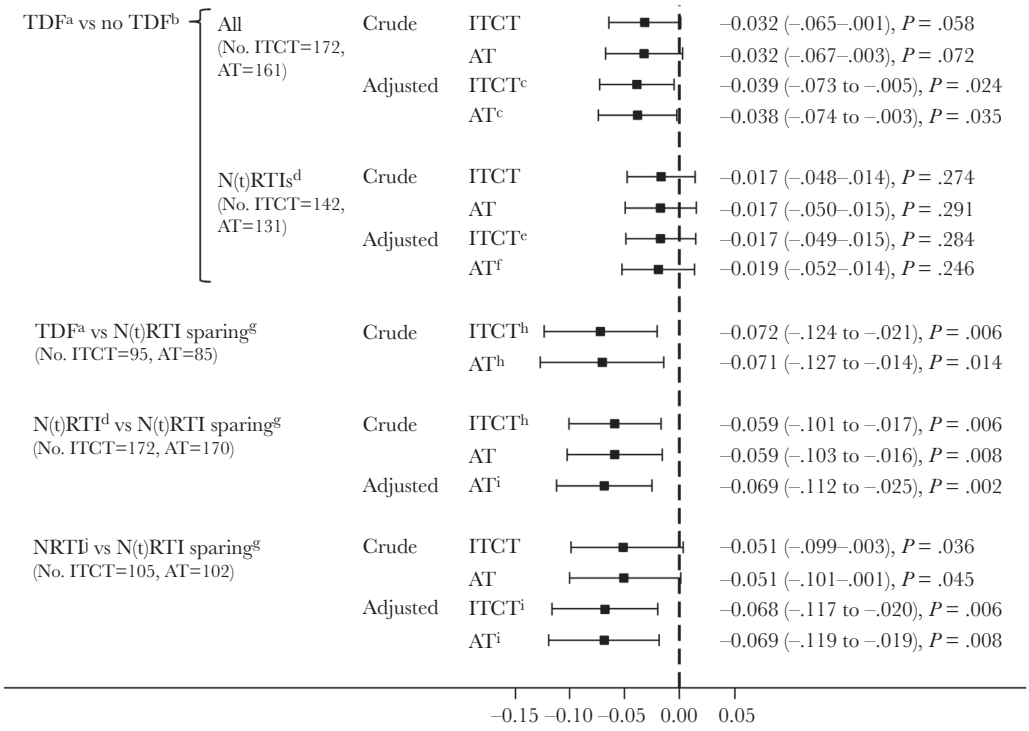

Figure 2. Mean difference in telomere length changes after 2 years, adjusted by telomere length at study entry. Telomere length was measured as mean ratio of the telomere amplification product to that of a single-copy gene. ABC, abacavir; AT, as-treated analysis (only participants without treatment changes at study entry); AZT, zidovudine; ddI, didanosine; FTC, emtricitabine; HIV, human immunodeficiency virus; ITCT, intention-to-continue-treatment analysis (ignoring treatment changes); N(t)RTI, nucleos(t)ide reverse transcriptase inhibitor; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine. [a]Regimens containing TDF. [b]Regimens not containing TDF. [c]Adjusted by active alcohol consumption. [d]Regimens containing N(t)RTIs or TDF (except 3TC alone). [e]Adjusted by age, income, time since HIV infection diagnosis, active alcohol and tobacco consumption, and active hepatitis C virus infection. [f]Adjusted by income, time since HIV infection diagnosis, active alcohol and tobacco consumption, study entry fibrinogen level, change in fibrinogen level during follow-up, and statin receipt. [g]Regimens without N(t)RTIs or with 3TC only. [h]No confounding variables were identified. Therefore, only the crude estimate is shown. [i]Adjusted by time since HIV infection diagnosis. [j]Regimens containing any nucleoside reverse transcriptase inhibitors (NRTIs; ie, ABC, AZT, ddI, 3TC, or FTC) except 3TC alone. A total of 69 participants received ABC/3TC, 3 received AZT/3TC, 1 received ABC/3TC/AZT, 1 received ABC, and 1 received ddI + 3TC.

associated with a smaller annual gain in TL. Exposure to tenofovir disoproxil fumarate at study entry was associated with a trend toward lower TL gains approaching statistical significance (P = .058). An unknown mechanism of HIV transmission was significantly associated with larger annual increases in blood TL.

In the multivariable analysis, women had significantly larger annual increases in blood TL, whereas time since HIV infection diagnosis and study entry exposure to N(t)RTI had a negative influence (Table 2).

DISCUSSION

We found that, after 2 years, participants treated with tenofovir disoproxil fumarate or abacavir had significantly lower blood TL gains than participants not receiving N(t)RTIs. This is the first prospective study showing that ART including some N(t)RTIs has a measurable negative effect on longitudinal blood TL changes of HIV-infected participants with virological suppression. Since shortened TL is one of the hallmarks of senescent T cells [12], these results suggest that ART regimens including tenofovir disoproxil fumarate or abacavir could play a role in delaying recovery from HIV-associated immunosenescence.

Our in vivo results reflect in vitro findings that tenofovir disoproxil fumarate and abacavir inhibit telomerase [7–9]. The largest difference in TL gain was between participants receiving the most potent inhibitor, tenofovir disoproxil fumarate, and participants receiving N(t)RTI-sparing regimens. When we compared regimens containing abacavir—a weaker inhibitor of telomerase—to N(t)RTI-sparing regimens, the point estimate for the difference in TL gain was smaller. When comparing tenofovir disoproxil fumarate–containing regimens to abacavir-containing regimens, the point estimate of the difference was even smaller and did not reach statistical significance. Taken together, these results point toward inhibition of telomerase as the underlying mechanism responsible for the TL differences observed in this cohort.

In a previous cross-sectional study [11], we did not find an association between history of tenofovir disoproxil fumarate exposure and blood TL. Importantly, in that study 43% of participants with a history of tenofovir disoproxil fumarate use had switched to boosted protease inhibitor monotherapy at the time of the cross-sectional analysis. In contrast, all tenofovir disoproxil fumarate–exposed participants in the present prospective

Table 2. Findings of Univariate and Multivariate Analyses of the Association Between Independent Factors and Annual Change in Telomere Length

	Univariate		Multivariate	
Factor	β (95% CI)	*P*	β (95% CI)	*P*
Age, per 10 y	−0.019 (−.037 to −.0003)	.046	…	
Father's age at birth, per 10 y	0.020 (−.004–.045)	.108	…	
Mother's age at birth, per 10 y	0.005 (−.022–.032)	.720	…	
Female sex	0.036 (−.001–.072)	.054	0.042 (.007–.077)	.019
Ethnicity other than white	0.014 (−.050–.078)	.667	..	
Education level				
Primary	Reference		…	
Secondary	0.018 (−.020–.056)	.346	…	
University	0.017 (−.023–.057)	.405	…	
Income level				
Low	Reference		…	
High	0.015 (−.018–.047)	.381	…	
Route of HIV transmission				
Sexual	Reference		…	
Parenteral	0.006 (−.029–.040)	.749	…	
Unknown	0.101 (.006–.197)	.037	…	
AIDS	0.002 (−.031–.034)	.916	…	
Time since HIV infection diagnosis, per 5 y	−0.013 (−.025–.0001)	.051	−0.018 (−.030 to −.005)	.006
N(t)RTI regimen at study entry[a]	−0.059 (−.101 to −.017)	.006	−0.070 (−.111 to −.029)	.001
TDF regimen at study entry[b]	−0.032 (−.065–.001)	.058	…	
Chronic kidney disease	0.021 (−.067–.108)	.642	…	
Hypertension	−0.007(−.047–.033)	.734	…	
Diabetes mellitus	−0.013 (−.059–.034)	.594	…	
Statin receipt	0.018 (−.017–.053)	.310	…	
HCV coinfection at study entry				
No	Reference		…	
Active	−0.030 (−.081–.020)	.239	…	
Past	0.009 (−.030–.048)	.650	…	
HCV infection cured during follow-up	−0.016 (−.075–.043)	.585	…	
Current tobacco use	0.011 (−.021–.043)	.486	…	
Current alcohol use	0.019 (−.014–.0508)	.257	…	
$CD4^+$ T-cell count, per 100 cells				
At study entry	−0.0002 (−.005–.004)	.939	…	
Change	−0.0001 (−.005–.005)	.957	…	
Glucose level, per 1 SD				
At study entry	−0.007 (−.026–.012)	.454	…	
Change	−0.003 (−.020–.015)	.774	…	
Creatinine level				
At study entry	−0.008 (−.022–.006)	.270	…	
Change	0.008 (−.007–.023)	.299	…	
Total cholesterol level, per 1 SD				
At study entry	0.014 (−.002–.029)	.081	…	
Change	−0.008 (−.024–.007)	.301	…	
HDL level, per 1 SD				
At study entry	0.010 (−.006–.027)	.204	…	
Change	0.006 (−.021–.009)	.428	…	
LDL level, per 1 SD				
At study entry	0.009 (−.006–.025)	.229	…	
Change	−0.001 (−.017–.015)	.928	…	
Triglycerides level, per 1 SD				
At study entry	−0.002 (−.017–.013)	.772	…	
Change	−0.004 (−.020–.011)	.569	…	
CRP level, per 1 SD				
At study entry	0.012 (−.006–.023)	.193	…	
Change	−0.010 (−.025–.005)	.178	…	

Table 2. Continued

	Univariate		Multivariate	
Factor	β (95% CI)	*P*	β (95% CI)	*P*
Fibrinogen level, per 1 SD				
At study entry	0.004 (−.012–.020)	.606	…	
Change	−0.016 (−.032–.0001)	.052	…	
D-dimer level, per 1 SD				
At study entry	0.007 (−.007–.022)	.322	…	
Change	−0.009 (−.024–.007)	.285	…	

Analyses were adjusted by telomere length at study entry. Variables with a *P* value of < .20 were entered in the saturated model for the multivariate analysis.

Abbreviations: CI, confidence interval; CRP, C-reactive protein; HCV, hepatitis C virus; HDL, high-density lipoprotein cholesterol; HIV, human immunodeficiency virus; LDL, low-density lipoprotein cholesterol; TDF, tenofovir disoproxil fumarate.

[a]Regimens containing TDF.

[b]Regimens containing any nucleos(t)ide reverse transcriptase inhibitor (N[t]RTI; except lamivudine alone) or TDF.

cohort were receiving tenofovir disoproxil fumarate at study entry. These results suggest that the negative impact of tenofovir disoproxil fumarate on blood TL changes might be reversible after stopping tenofovir disoproxil fumarate treatment.

Our results also contradict those from a substudy of the MONET clinical trial that compared darunavir/ritonavir monotherapy to darunavir/ritonavir and 2 N(t)RTIs for maintenance of virological suppression [13] and found that continuation of N(t)RTI treatment was not associated with TL changes. However, the MONET substudy was underpowered to find an impact of N(t)RTIs on TL changes: the sample size was 124 participants, from whom samples were obtained after week 48 from only 88%, there was an imbalance at study entry, with longer TL in participants assigned to stop N(t)RTI therapy. In addition, statistical analysis did not adjust for study entry TL, as recommended for longitudinal TL studies [14].

We found that, overall, mean blood TL increased after 2 years regardless of ART strategy. This is contrary to studies performed in the general population, in which mean blood TL measured by PCR analysis showed annual decreases in blood TL [15]. This difference could imply that, despite the high $CD4^+$ T-cell counts and long duration of viral suppression, our participants are still experiencing immune reconstitution. We think it is plausible that our subgroup of participants with increases in blood TL might be shifting their T-lymphocyte subpopulations toward less mature T-cell phenotypes with longer TL [16, 17], thus translating into the increase in mean blood TL. We hypothesize that tenofovir disoproxil fumarate or abacavir can interfere with this process of immune reconstitution. It is also possible that participants receiving tenofovir disoproxil fumarate or abacavir have a similar distribution of T-lymphocyte subpopulations but with overall shorter TL. Interestingly, Cobos Jimenez et al [18] have shown that HIV-infected participants with virological suppression have shorter TL in PBMCs than well-matched controls, despite a similar distribution of senescent T cells.

This was not a randomized study, and as such, we cannot determine whether unmeasured variables could influence blood TL change. We performed analysis of multiple subgroups, increasing the likelihood of finding spurious associations with a *P* value of < .05. However, effects in all groups were highly significant and consistent with our a priori hypothesis based on in vitro data, leading us to believe that the observed associations corresponded to true effects. Another limitation is that we did not determine TL on specific subsets of T cells. Consequently, we cannot at this time prove our hypothesis that blood TL changes are driven by modifications in T-cell subpopulations. The best way to avoid some of these limitations would be to measure TL changes in different T-cell subpopulations in randomized clinical trials aiming to compare ART including 2 N(t)RTIs to N(t)RTI-sparing regimens [19, 20].

The clinical relevance of the differences in blood TL found in this cohort is unknown. One recent study in 51 injection drug users showed that, 3 months after HIV seroconversion, the TL in PBMCs measured by quantitative PCR analysis decreased 13% [3]. In our study, the mean adjusted per-protocol difference in TL gain between participants receiving and those not receiving tenofovir disoproxil fumarate at week 96 was 0.0384, representing a 3% decrease from the blood TL at study entry in the group not exposed to tenofovir disoproxil fumarate. We consider that this difference between groups is not negligible. Additionally, the differences among regimens increased at follow-up, suggesting a cumulative impact of N(t)RTIs on TL. Currently, TL is not a prognostic biomarker in persons living with HIV. However, as repeatedly reported [21, 22], TL correlates with immunosenescence in HIV-infected individuals, so the prognostic importance of blood TL in persons living with HIV could become an interesting subject of research.

The results found in this cohort of HIV-infected participants with >8 years of virological suppression are in contrast with our results from the NEAT 001 clinical trial substudy [23]. In NEAT 001, ART-naive participants treated with tenofovir disoproxil fumarate/emtricitabine and ritonavir-boosted darunavir had significant higher gains in blood TL after 2 years, compared with participants receiving a N(t)RTI-sparing regimen of raltegravir

and ritonavir-boosted darunavir. Why did tenofovir disoproxil fumarate have a negative impact on blood TL changes in our prospective cohort of participants with virological suppression but a positive impact 2 years after starting ART among participants in NEAT 001? Our hypothesis is that, in NEAT 001, the main driver of changes in blood TL was initial control of HIV replication, while in the present prospective cohort of aviremic participants, the main drivers of TL changes are both control of HIV replication and telomerase inhibition caused by tenofovir or abacavir.

Compared with tenofovir disoproxil fumarate and emtricitabine, both darunavir and raltegravir have lower concentrations in lymph node tissue [24–26]. We think that, because of these low tissue levels, participants treated with darunavir and raltegravir had a slower decay of HIV replication in lymph nodes and a persistent stimulus for T cells to differentiate into mature phenotypes with shorter TL. In contrast to NEAT 001, participants in our prospective cohort have sustained control of HIV replication. We believe it is in the scenario of prolonged suppression of viremia that inhibition of telomerase caused by tenofovir or abacavir could be most apparent and result in lower blood TL gains, as it is observed in our cohort.

In summary, in this cohort of HIV-infected participants with long-standing virological suppression, ART, regardless of the regimen, had an overall positive impact on sequential TL changes. However, N(t)RTI-sparing ART was associated with larger gains in blood TL than ART regimens containing tenofovir disoproxil fumarate or abacavir. Further research is needed to confirm whether long-term treatment with these N(t)RTIs disturbs recovery from HIV-related immunosenescence.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgment. We are grateful to all study participants.

Financial support. This work was supported by the Fondo de Investigaciones Sanitarias, Instituto de Salud Carlos III (grants PI13/01467 and PI14/01495 [supported by the European Regional Development Fund], Rio Hortega fellowship [to R. d. M.], and predoctoral fellowship [to N. S.-A.]).

Potential conflicts of interest. R. M. has received payment for development of educational lectures from Janssen. N. S.-A. has received personal fees from Janssen-Cilag and from Gilead outside the submitted work. J. I. B. has received payment for lectures, including service on the speaker's bureaus, from ViiV Healthcare and Janssen; and currently receives payment for expert testimony, from Gilead Sciences and Merck Sharp and Dohme. I. P.-V. has received payment for consultancies, from Janssen-Cilag, Gilead, and ViiV Healthcare, and for lectures, including service on speaker's bureaus, from Merck Sharp & Dohme. M. L.-M. is receiving payment for board membership, from Janssen-Cilag and Abbvie, and for development of educational presentations, from Janssen-Cilag, Abbvie, and Gilead; and has received payment for consultancies, from Janssen Cilag, Abbvie, and ViiV Healthcare; for expert testimony, from Abbvie and Janssen-Cilag; and for lectures, including service on speaker's bureaus, from Janssen-Cilag, ViiV Healthcare, and Abbvie. J. R. A. is currently receiving payment for board membership and consultancies, from ViiV Healthcare, Janssen-Cilag, Gilead and Merck Sharp & Dohme; and is receiving payment for lectures, including service on speaker's bureaus, from ViiV Healthcare, Janssen Cilag, Gilead, and Merck Sharp & Dohme. All other authors report no potential conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Material suplementario

Table S1a.Blood telomere length (TL) at baseline and after 2 years of follow-up, by regimen. (Intention to continue treatment analysis)

		TDF N=67	p[1]	p[2]	N(t)RTI N=142	p[3]	NRTI N=75	p[4]	Non-TDF N=105	N(t)RTI sparing N=30
Baseline	Mean TL (SD)	1.21 (0.28)	0.014	0.071	1.24 (0.30)	0.026	1.27 (0.36)	0.109	1.30 (0.32)	1.38 (0.35)
	Mean dispersion of measures (SD)	0.06 (0.03)	0.030	0.073	0.06 (0.04)	0.122	0.06 (0.04)	0.382	0.07 (0.04)	0.07 (0.04)
Follow-up	Mean TL (SD)	1.24 (0.24)	<0.001	0.009	1.28 (0.25)	0.001	1.31 (0.25)	0.012	1.35 (0.28)	1.46 (0.31)
	Mean dispersion of measures (SD)	0.05 (0.03)	0.884	0.053	0.05 (0.03)	0.349	0.06 (0.03)	0.095	0.06 (0.03)	0.05 (0.03)
Change (follow-up vs. baseline). Mean (95% CI)		0.028 (-0.028 to 0.085) p*=0.312	0.423	0.586	0.034 (-0.003 to 0.072) p*=0.072	0.404	0.040 (-0.012 to 0.091) p*=0.131	0.517	0.050 (-0.001 to 0.101) p*=0.054	0.077 (-0.053 to 0.206) p*=0.236
Mean annual change (corrected for varying intervals between baseline and follow-up). Mean (95% CI)		0.013 (-0.015 to 0.041) p*=0.357	0.345	0.562	0.016 (-0.003 to 0.034) p*=0.104	0.302	0.018 (-0.008 to 0.043) p*=0.173	0.401	0.025 (-0.001 to 0.051) p*=0.063	0.042 (-0.025 to 0.109) p*=0.212
N (%) with TL lengthening in at follow-up		41 (61.2)	0.606	0.975	85 (59.9)	0.487	44 (58.7)	0.448	64 (60.9)	19 (67.9)

Table S1b. Blood telomere length at baseline and after 2 years of follow-up, by regimen. (As treated analysis)

		TDF N=57	p[1]	p[2]	N(t)RTI N=142	p[3]	NRTI N=74	p[4]	Non-TDF N=104	N(t)RTI sparing N=28
Baseline	Mean length (SD)	1.20 (0.29)	0.016	0.060	1.24 (0.30)	0.029	1.27 (0.31)	0.124	1.30 (0.33)	1.38 (0.36)
	Mean dispersion of measures (SD)	0.06 (0.03)	0.060	0.103	0.06 (0.04)	0.176	0.06 (0.04)	0.446	0.07 (0.04)	0.07 (0.04)
Follow-up	Mean length (SD)	1.23 (0.26)	0.001	0.009	1.28 (0.25)	0.001	1.31 (0.25)	0.016	1.35 (0.27)	1.46 (0.32)
	Mean dispersion of measures (SD)	0.05 (0.03)	0.651	0. 085	0.05 (0.03)	0.562	0.06 (0.03)	0.210	0.06 (0.03)	0.05 (0.03)
Change (follow-up vs. baseline). Mean (95% CI)		0.031 (-0.028 to 0.090) p*=0.302	0.476	0.631	0.034 (-0.003 to 0.072) p*=0.072	0.428	0.040 (-0.012 to 0.092) p*=0.133	0.547	0.051 (-0.001 to 0.102) p*=0.055	0.076 (-0.064 to 0.215) p*=0.274
Mean annual change (corrected for varying intervals between baseline and follow-up). Mean (95% CI)		0.014 (-0.015 to 0.044) p*=0.335	0.408	0.623	0.016 (-0.003 to 0.034) p*=0.104	0.327	0.018 (-0.008 to 0.044) p*=0.175	0.437	0.025 (-0.001 to 0.044) p*=0.063	0.041 (-0.031 to 0.114) p*=0.249
N (%) with TL lengthening at follow-up		35 (61.4)	0.797	0.918	85 (59.9)	0.661	43 (58.1)	0.570	63(60.6)	18 (64.3)

*p-value for the null hypothesis of change in telomere length=0;[1]p-value comparing TDF vs. N(t)RTI sparing; [2]p-value comparing TDF vs. non-TDF.[3] p-value comparing N(t)RTI vs N(t)RTI sparing; [4]p-value comparing NRTI vs. N(t)RTI sparing.

TDF: Regimens containing Tenofovir Disoproxil Fumarate. No-TDF: Regimens not containing Tenofovir Disoproxil Fumarate N(t)RTI: Regimens containing any Nucleoside Reverse Transcriptase Inhibitors or TDF (except 3TC alone). NRTI: Regimens containing any Nucleoside Reverse Transcriptase Inhibitors (ABC, AZT, ddI, 3TC or FTC). 3TC alone excluded. No TDF: Regimens not including TDF. N(t)RTI sparing: Regimens without N(t)RTIs or with only 3TC

Table S2. Effect of treatment with TDF and NRTI on change of telomere length. Change in length adjusted by baseline length.

		TDF vs. non-TDF (All)		TDF vs. non-TDF [in those on N(t)RTI]		TDF vs. N(t)RTI sparing		N(t)RTI vs. N(t)RTI sparing		NRTI vs. N(t)RTI sparing	
		Coef. (95%CI)	p-val	Coef. (95%CI)	p-val	Coef. (95%CI)	p-val	Coef. (95%CI)	p-val	Coef. (95%CI)	p-val
Intention-to continue treatment	N	172		142		97		172		105	
	Crude	-0.0319 (-0.0648 to 0.010)	0.058	-0.0171 (-0.0479 to 0.0137)	0.274	-0.0722 (-0.1237 to -0.0207)	0.006	-0.0592 (-0.1011 to -0.0172)	0.006	-0.0513 (-0.0991 to 0.0034)	0.036
	Adjusted	-0.0391 (-0.0729 to -0.0053)[a]	0.024	-0.0174 (-0.0493 to 0.0146)[b]	0.284	-	-	-	-	-0.0682 (-0.1166 to -0.0198)[d]	0.006
As treated	N	161		131		85		170		102	
	Crude	-0.0322 (-0.0673 to 0.0029)	0.072	-0.0174 (-0.0499 to 0.0151)	0.291	-0.0707 (-0.1270 to -0.0144)	0.014	-0.0593 (-0.1027 to -0.0159)	0.008	-0.0508 (-0.10051 to 0.0011)	0.045
	Adjusted	-0.0384 (-0.0739 to -0.0028)[a]	0.035	-0.0194 (-0.0524 to 0.0136)[b]	0.246	-	-	-0.0689 (-0.1122 to -0.0255)[c]	0.002	-0.0690 (-0.1194 to -0.0187)[d]	0.008

[a]Intention-to-treat (ITT) and per-protocol (PP) analyses adjusted by active alcohol consumption; [b] Both ITT and PP analyses adjusted by income, time since HIV diagnosis and active alcohol and tobacco consumption, ITT additionally adjusted by age and active HCV infection, PP additionally adjusted by baseline fibrinogen and change in fibrinogen during follow-up and statins intake; [c] PP analysis adjusted by time since HIV diagnosis; [d]Both ITT and PP analyses adjusted by time since HIV diagnosis.

Table S3. Antiretroviral Regimens

ART REGIMEN	N (%)
TDF/FTC/EFV	52 (30.23)
ABC/3TC EFV	15 (8.72)
ABC/3TC ATV	14 (8.14)
DRV/r	13 (7.56)
ABC/3TC NEV	10 (5.81)
ABC/3TC RAL	9 (5.23)
ABC/3TC ETV	8 (4.65)
LPV/r	8 (4.65)
ABC/3TC LPV/r	5 (2.91)
TDF/FTC/RIL	4 (2.33)
ABC/3TC FAMP/r	3 (1.74)
3TC DRV/r	2 (1.16)
ABC/3TC DTG	2 (1.16)
ABC/3TC RIL	2 (1.16)
AZT/3TC LPV/r	2 (1.16)
TDF/FTC ATV/r	2 (1.16)
3TC ATV RAL	1 (0.58)
3TC ETV RAL	1 (0.58)
3TC LPV/r	1 (0.58)
3TC TDF EFV	1 (0.58)
3TC TDF LPV/r	1 (0.58)
3TC TDF d4T	1 (0.58)
3TC ddI EFV	1 (0.58)
ABC ATV RAL	1 (0.58)
ABC/3TC DRV/r	1 (0.58)
ABC/3TC/AZT	1 (0.58)

ATV/r	1 (0.58)
AZT/3TC RAL	1 (0.58)
ETV LPV/r	1 (0.58)
ETV RAL	1 (0.58)
LPV/r RAL	1 (0.58)
TDF/FTC ATV	1 (0.58)
TDF/FTC DRV/r RAL	1 (0.58)
TDF/FTC FAMP RIT	1 (0.58)
TDF/FTC LPV/r	1 (0.58)
TDF/FTC NEV	1 (0.58)
TDF/FTC RAL	1 (0.58)
Total	172 (100)

3TC: lamivudine, ABC: abacavir, ATV: atazanavir, AZT: zidovudine, d4T: stavudine, ddI: didanosine, DRV: darunavir, DTG: dolutegravir, EFV: efavirenz, ETV: etravirine, FAMP: fosamprenavir, FTC: emtricitabine, LPV/r: lopinavir + ritonavir, NEV: nevirapine, RAL: raltegravir, RIL: rilpivirine, RIT: ritonavir, SAQ: saquinavir, TDF: tenofovir disoproxil fumarate

Publicación 4

BLOOD TELOMERE LENGTH CHANGES AFTER RITONAVIR-BOOSTED DARUNAVIR COMBINED WITH RALTEGRAVIR OR TENOFOVIR–EMTRICITABINE IN ANTIRETROVIRAL-NAIVE ADULTS INFECTED WITH HIV-1

Los estudios previos *in vivo* presentados en esta tesis se han realizado en la cohorte de pacientes VIH+ con prolongada supresión virológica del Hospital Universitario La Paz. Dado que los estudios de cohortes no son aleatorizados, no se puede determinar si las variables que no han sido medidas podrían influir en el cambio de la LT en sangre. Además, para determinar el impacto de los N(t)RTIs un mejor grupo de estudio son los pacientes naïve VIH+. Por lo tanto, el presente trabajo se ha realizado como un subestudio del ensayo clínico NEAT 001/ ANRS 143, en el cual los pacientes fueron aleatorizados a empezar TAR con una pauta que incluía dos N(t)RTIs (Darunavir/ritonavir (DRV/r) + TDF/FTC) o con una pauta de TAR ahorradora de N(t)RTIs (DRV/r+ Raltegravir (RAL)).

En este estudio longitudinal, en el cual se ha evaluado el cambio de la LT en los pacientes naïve tras dos años de tratamiento, los resultados obtenidos indican que en este caso la pauta de TAR con TDF/FTC se asocia a una mayor ganancia de la LT en sangre que la pauta ahorradora de N(t)RTIs, lo que sugiere una mejor recuperación inicial de la inmunosenescencia asociada al VIH.

Mi contribución personal en la parte experimental de este trabajo consistió en el procesamiento de las muestras de sangre y la determinación de la LT en las mismas. En cuanto a la elaboración del manuscrito he participado en el análisis de los resultados obtenidos, el diseño de figuras y tablas y la escritura del artículo.

The Journal of Infectious Diseases

MAJOR ARTICLE

Blood Telomere Length Changes After Ritonavir-Boosted Darunavir Combined With Raltegravir or Tenofovir-Emtricitabine in Antiretroviral-Naive Adults Infected With HIV-1

Natalia Stella-Ascariz,[1,a] Rocio Montejano,[1,a] Javier Rodriguez-Centeno,[1] Belen Alejos,[2] Christine Schwimmer,[3] Jose I. Bernardino,[1] Berta Rodes,[1] Clotilde Allavena,[4] Christian Hoffmann,[5] Magnus Gisslén,[6] Rosa de Miguel,[1] Andrés Esteban-Cantos,[1] Cédrick Wallet,[3] François Raffi,[7] Jose R. Arribas;[1] and the NEAT 001/ ANRS 143 Study Group

[1]Hospital La Paz Institute for Health Research, and [2]Institute of Health Carlos III, Madrid, Spain; [3]University of Bordeaux, Inserm, Bordeaux Population Health Research Center, UMR 1219, France; [4]Hôtel-Dieu and Centre d'Investigation Clinique Unité d'Investigation Clinique 1413 Inserm, Centre Hospitalier Universitaire, Nantes, France; [5]Infektionsmedizinisches Centrum Hamburg Study Center, Germany; [6]Sahlgrenska Academy at the University of Gothenburg, Sweden; and [7]Centre Hospitalier Universitaire de Nantes, France

(See the Major Article by Montejano et al on pages 1531–40 and Editorial Commentary by Côté and Hsieh on pages 1521–2.)

Background. Tenofovir is a potent inhibitor of human telomerase. The clinical relevance of this inhibition is unknown.

Methods. NEAT001/ANRS143 is a randomized trial that showed noninferiority over 96 weeks of ritonavir-boosted darunavir plus raltegravir versus tenofovir disoproxil fumarate/emtricitabine in 805 antiretroviral antiretrovrial-naive HIV-infected adults. We compared changes in whole-blood telomere length measured with quantitative polymerase chain reaction in 201 randomly selected participants (104 raltegravir and 97 tenofovir disoproxil fumarate/emtricitabine). We performed multivariable estimative and predictive linear regression.

Results. At week 96, participants receiving tenofovir disoproxil fumarate/emtricitabine had a statistically significant higher gain in telomere length than participants receiving raltegravir. Difference in mean telomere length change between groups (tenofovir disoproxil fumarate/emtricitabine minus raltegravir) from baseline to week 96 adjusted by baseline telomere length was 0.031 ($P = .009$). This difference was not significantly confounded by age, gender, known duration of HIV infection, CD4 (baseline/nadir), CD8 cells, CD4/CD8 ratio, HIV viral load (baseline/week 96), tobacco and alcohol consumption, statins, or hepatitis C.

Conclusion. Antiretroviral-naive HIV-infected adults receiving ritonavir-boosted darunavir and tenofovir disoproxil fumarate/emtricitabine had a significant higher gain in blood telomere length than those receiving ritonavir-boosted darunavir and raltegravir, suggesting a better initial recovery from HIV-associated immunosenescence.

Keywords. HIV infection; antiretroviral therapy; darunavir/ritonavir; raltegravir; tenofovir; telomere length; telomerase.

Human immunodeficiency virus (HIV) infection leads to an accelerated immunosenescence status marked by dominant senescent and exhausted phenotypes of mature T cells with a decrease in naive T cells [1, 2]. Senescent T cells have limited proliferative capacity due to telomere attrition [3]. In keeping with this immunosenescence status, HIV-infected individuals have shorter blood telomere length (TL) than HIV-uninfected controls [4–7].

Antiretroviral treatment (ART) partially reverses HIV-associated immunosenescence. Initial control of HIV replication translates into an increase in naive and central memory CD4 and CD8 cells that have longer telomeres. The increase in TL after initiating ART is correlated mainly with shifts of CD8 cells subpopulations towards less mature phenotypes [8, 9].

In vitro studies have shown that tenofovir and abacavir, 2 recommended nucleos(t)ide reverse transcriptase inhibitors (N(t)RTI), are able to inhibit human telomerase, tenofovir being the most potent inhibitor [10–12]. The clinical relevance of this in vitro finding is unknown. There are no studies comparing TL changes in ART-naive participants who start treatment with N(t)RTI-containing versus N(t)RTI-sparing ART. For this reason, we have evaluated blood TL changes in a substudy of the NEAT001/ANRS 143 clinical trial that compared ritonavir-boosted darunavir combined with raltegravir or tenofovir disoproxil fumarate/emtricitabine in ART-naive adults. Our research hypothesis was

Received 26 June 2018; editorial decision 30 April 2018; accepted 27 June 2018; published online July 3, 2018.

Presented in part: Conference on Retroviruses and Opportunistic Infections, Boston 4–7 March 2018. Abstract 1589. Stella-Ascariz N, et al. Blood telomere length changes after DRV/R + either RAL or TDF/FTC as first line ART.

[a]N. S.-A. and R. M. contributed equally.

Correspondence: J. R. Arribas, MD, Hospital La Paz, IdiPAZ Edificio de Investigación, Despacho 3.3, Paseo de la Castellana 261. 28046, Madrid, Spain (joser.arribas@salud.madrid.org).

DOI: 10.1093/infdis/jiy399

that exposure to tenofovir, in line with its in vitro activity inhibiting the telomerase, would have a negative impact on blood TL changes.

METHODS

Study Design and Participants

NEAT001/ANRS143 was a randomized 1:1, open-label, 96 week, noninferiority trial conducted in 78 clinical sites in 15 European countries between August 2010 and October 2013. Ethics committee and competent authority approval was obtained for all participating centers, in accordance with the principles of the Declaration of Helsinki. Inclusion criteria were: HIV RNA greater than 1000 copies per mL and CD4 cell count under 500 cells per μL in ART-naive participants (the full study design and patient population have been previously described) [13]. Participants were excluded if they presented any of the following: treatment for malignant disease, positive hepatitis B virus surface antigen, pregnancy, creatinine clearance of less than 60 mL/min or any other relevant laboratory abnormalities.

Randomization and Masking

Randomization of the parent study was performed as previously reported [13]. We randomly assigned participants (1:1) to receive oral treatment with either 800 mg darunavir and 100 mg ritonavir once per day plus 400 mg raltegravir twice daily (N(t)RTI-sparing regimen) or 800 mg darunavir and 100 mg ritonavir plus tenofovir disoproxil fumarate 245 mg and 200 mg emtricitabine in a fixed-dose combination once per day (standard regimen). Tenofovir disoproxil fumarate/emtricitabine was provided by Gilead Sciences, darunavir by Janssen Pharmaceuticals, and raltegravir by Merck Laboratories. Participants included in this substudy were randomly selected stratified by treatment group using Stata software (version 14.0; Stata Corporation, College Station, TX).

Participants were applicable for enrolment in this substudy if they had consented to biobank preservation and had available blood samples from both baseline and week 96. Of the total of 805 participants enrolled in NEAT 001, 681 participants had appropriate blood samples at both baseline and 96 weeks. Because there were no prior data on which to base sample size calculation, of these 681 participants we selected an initial random sample of 100 subjects for TL determinations. This initial blinded comparison revealed significant differences between groups (Supplementary Table 1). In order to have a more precise estimate of the differences and to confirm our findings, the sample size was further increased afterwards with an additional randomly selected 100 subjects. We obtained written informed consent from all participants for the parent study.

Telomere Length Determination by Quantitative Real-Time PCR

Genomic DNA was isolated from whole blood using QIAamp DNA Blood Mini Kit (Qiagen) according to manufacturer's instructions. Relative telomere length, expressed as ratio of telomere (T) to single-copy gene (S), was determined by monochrome quantitative multiplex polymerase chain reaction (PCR) assay, with minor modifications as described in our prior study [14]. A standard curve was prepared with genomic DNA from a pool of 3 healthy volunteers by serial dilution and was included in triplicate in each run together with a reference sample and negative control. Baseline and follow-up samples were assayed in triplicate on the same PCR plate and those with a coefficient of variation (CV) greater than 10% were reanalyzed. The intra-assay CV was calculated as the average of the individual CVs of the samples and the interassay CV using the reference sample. The intra-assay coefficient of variation for T/S ratio, T-cycle threshold and S-cycle threshold were 4.7%, 0.4%, and 0.3%, and the interassay CV were 5.7%, 1.0%, and 0.8%, respectively

Outcomes

The primary outcome of the telomere substudy was the change in TL from baseline to week 96. The secondary outcomes were increase in the TL and increase greater than 1 standard deviation (SD) in TL from baseline to week 96.

Covariates included in the analysis were gender (male, female), age, ethnicity (Asian, Black, Caucasian, other), and alcohol (non/moderate, exdrinker, current drinker, unknown) and tobacco (never, stopped, currently) consumption at baseline, hepatitis C virus (HCV) coinfection (negative, nonactive, active, unknown), statin treatment (yes, no), HIV mechanism of acquisition (homo/bisexual, heterosexual, others, unknown), time since HIV diagnosis, HIV CDC clinical stage (A, B, C), CD4 cell count, nadir CD4 cell count, CD8 cell count, CD4/CD8 ratio, $\log_{10}$ HIV-1 RNA, HIV-1 RNA (<100 000 HIV-RNA copies/mL, ≥100 000 HIV-1 RNA copies/mL), and baseline TL.

Statistical Analyses

Characteristics of the participants with available samples (n = 681) and those included in the substudy (n = 201) were described using absolute and relative frequencies and mean (standard deviation) for categorical and continuous variables, respectively. Balance in the treatment group was analyzed using X^2 or median tests accordingly. We calculated the mean increases and 95% confidence intervals in TL from baseline to week 96. We performed a multivariable estimative linear regression model to assess differences in TL increases by treatment group adjusting for baseline TL and potential confounders (retaining in the model variables that produced a change ≥15% in the treatment mean difference). We also performed a multivariable predictive linear regression model to identify predictive factors for TL change. All variables that retained a significant independent association ($P < .01$) were included in the final model. We used multivariable logistic regression models to assess differences by treatment group in the proportion of participants attaining a TL increase and a TL increase >1 SD from baseline to week 96.

As a complementary analysis, we used Kaplan-Meier method to calculate probability of reaching virological response (HIV-1 RNA < 50 copies/mL) and CD4/CD8 ratio normalization (CD4/CD8 > 0.4). Wald tests were used to derive *P* values. All statistical analyses were performed using Stata software (version 14.0; Stata Corporation, College Station, TX).

RESULTS

Characteristics of Study Participants

Telomere length was analyzed on 201 random participants, 97 in the tenofovir disoproxil fumarate/emtricitabine exposed group and 104 in the raltegravir exposed group. Baseline characteristics were all well balanced between groups, with no notable differences from the total telomere substudy samples (Table 1). The great majority of participants were male. There were no statistically significant differences in gender, age, or race. Both groups had acquired HIV mainly by sexual transmission and the mean time since HIV diagnosis was 2.1 years. We did not find differences in tobacco or alcohol consumption. There were no statistically significant differences in CD4 cell count, CD4 nadir, CD8 cell count, CD4/CD8 ratio, HIV-1 RNA load, HCV coinfection, or treatment with statins.

At week 96, we did not find differences between groups in immunovirologic response (Table 2). More than 90% of participants had achieved virological suppression in both treatment arms. There were not statistically significant differences in the proportion of participants reaching the primary endpoint of the core trial (virologic nonresponse or failure, death AIDS event, or serious non-AIDS event). The number of virological endpoints was the same in each arm. Time to virological response was significantly shorter for participants randomized to raltegravir (Table 2 and Supplementary Figure 1). There were no statistically significant differences in any of the immunological parameters measured at week 96: CD4 change, CD8 change, CD4/CD8 change, proportions with CD4/CD8 above 0.4 or 1, and time to achieve a CD4/CD8 ratio >0.4 (Table 2 and Supplementary Figure 2).

Blood Telomere Length Changes

Blood telomere length analysis showed that at baseline there were no statistically significant differences between groups. After 96 weeks, both groups had a gain in TL: mean TL in the total analyzed samples increased by 0.028 (Figure 1 and Supplementary Table 2). However, the increase in TL was only significant in the tenofovir disoproxil fumarate/emtricitabine group. Additionally, the proportion of participants who had increase in TL at follow-up was 71% in tenofovir disoproxil fumarate/emtricitabine group versus 57% in the raltegravir group.

Multivariable Estimative Analysis

In our estimative analysis, exposure to tenofovir disoproxil fumarate/emtricitabine had a positive impact on TL change. After 96 weeks, tenofovir disoproxil fumarate/emtricitabine–exposed participants had a gain in mean blood TL adjusted by baseline TL that was 0.031 superior to raltegravir-exposed participants ($P = .009$) (Figure 2). This effect was not significantly confounded by age, gender, race, time since HIV diagnosis, baseline HIV RNA, nadir or baseline CD4 cell count, baseline CD8, baseline CD4/CD8, tobacco and alcohol consumption, statins, or hepatitis C. These results were unchanged when TL was analyzed as a binary variable (TL shortened/not shortened; data not shown).

Predictive Model

In the univariate analysis treatment with tenofovir disoproxil fumarate/emtricitabine, younger age and no current alcohol consumption were significantly associated with a gain in mean TL among all participants. We found no significant associations with tobacco, gender, race, or statin treatment. Regarding HIV-related factors, we found no significant associations of mean TL gain with time since HIV diagnosis, nadir or baseline CD4, baseline HIV RNA, and HCV coinfection (Table 3).

In the multivariable analysis, independent predictors of gain in TL were baseline TL ($P < .001$), treatment with tenofovir disoproxil fumarate/emtricitabine ($P = .005$), and no current alcohol consumption at baseline ($P = .026$). There was a trend ($P = .097$) towards younger age also being associated with higher gains in TL (Table 3).

DISCUSSION

Surprisingly, and contrary to our research hypothesis based on in vitro study results [10–12], participants receiving ritonavir-boosted darunavir, emtricitabine, and tenofovir disoproxil fumarate had significantly higher gains in TL than those receiving a N(t)RTI-sparing regimen (ritonavir-boosted darunavir and raltegravir). This is the first clinical trial showing that N(t)RTI-containing ART has a measurable positive impact on longitudinal TL changes, in naive HIV participants. This result suggests that ART regimens including N(t)RTIs could play an important role in initial recovery from HIV-associated immunosenescence.

Although overall mean blood TL increased after 2 years regardless of ART, this was only statistically significant in participants randomized to tenofovir disoproxil fumarate/emtricitabine. The difference in mean gain in TL was also statistically significant in favor of tenofovir disoproxil fumarate/emtricitabine, and the proportion of participants with increases in TL was 14% higher than the N(t)RTI-sparing group. In our estimative analysis the adjusted difference between groups in mean TL changes was 0.031, which represents 4.2% of the baseline mean blood TL for the whole population. One recent study in 51 intravenous drug users showed that 3 months after HIV seroconversion the TL in peripheral blood mononuclear cells measured by quantitative PCR decreased 13% [15]. Therefore,

Table 1. Baseline Characteristics of Study Participants

	RAL+DRV/r (n = 104)	TDF/FTC+ DRV/r+ (n = 97)	*P* Value
Gender, n (%)			.862
Female	11 (10.6)	11 (11.3)	
Age, median years (IQR)	37.6 (30.5–46.3)	37.3 (30.7–46.4)	.722
Ethnicity, n (%)			.206
Asian	2 (1.9)	1 (1.0)	
Black	11 (10.6)	12 (12.4)	
Caucasian	90 (86.5)	78 (80.4)	
Other	1 (1.0)	6 (6.2)	
Mode HIV transmission, n (%)			.691
Homosexual/bisexual	72 (69.2)	69 (71.1)	
IVDU (since 1984)	0 (0.0)	1 (1.0)	
Heterosexual	24 (23.1)	22 (22.7)	
Other	1 (1.0)	0 (0.0)	
Unknown	7 (6.7)	5 (5.2)	
Smoking, n (%)			.417
Never	61 (58.7)	48 (49.5)	
Stopped	8 (7.7)	10 (10.3)	
Currently	35 (33.7)	39 (40.2)	
Alcohol, n (%)			.197
Non/moderate drinker	99 (95.2)	86 (88.7)	
Exdrinker	1 (1.0)	1 (1.0)	
Current drinker	4 (3.8)	10 (10.3)	
Time since HIV diagnosis, median years (IQR)	1.3 (0.4–2.7)	1.4 (0.2–2.8)	.887
HIV CDC clinical stage, n (%)			.455
A	87 (83.7)	87 (89.7)	
B	12 (11.5)	7 (7.2)	
C	5 (4.8)	3 (3.1)	
CD4 cell count, cells per μL, n (%)			.866
<50	5 (4.8)	3 (3.1)	
50–199	14 (13.5)	12 (12.4)	
200–349	36 (34.6)	40 (41.2)	
150–499	42 (40.4)	37 (38.1)	
≥500	7 (6.7)	5 (5.2)	
CD4 cell count, median cells per μL (IQR)	346 (267–425)	333 (246–400)	.609
Nadir CD4 cell count, median cells per μL (IQR)	333 (245–394)	300 (232–360)	.069
CD8 cell count, median cells per μL (IQR)	886 (656–1205)	839 (622–1085)	.276
CD4/CD8 ratio, median (IQR)	0.4 (0.2–0.5)	0.4 (0.3–0.5)	.561
HIV-1 RNA, median log10 cop/ mL), (IQR)	4.7 (4.5–5.2)	4.7 (4.5–5.1)	.834
HIV-1 RNA, n (%)			.568
<100 000 copies/mL	69 (66.3)	68 (70.1)	
≥100 000 copies/mL	35 (33.7)	29 (29.9)	
HCV coinfection, n (%)			.472
Negative	103 (99.0)	94 (96.9)	
Nonactive	1 (1.0)	2 (2.1)	
Active	0 (0.0)	1 (1.0)	
Statins, n (%)	9 (8.7)	7 (7.2)	.707

Abbreviations: DRV/r, darunavir/ ritonavir; FTC, emtricitabine; IQR, interquartile range; IVDU, intravenous drug use; RAL, raltegravir; TDF, tenofovir disoproxil fumarate.

it is likely that the difference between arms in recovery of blood TL after starting ART is important.

The positive impact of tenofovir disoproxil fumarate/emtricitabine on TL gain was not confounded by baseline or week 96 variables. In our predictive analysis the only factors associated with a statistically higher gain in TL were tenofovir disoproxil fumarate/emtricitabine and no current alcohol consumption, with younger age approaching significance. Alcohol abuse has been previously associated with TL attrition in HIV-negative individuals [16].

To the best of our knowledge this is the first clinical trial that compares TL changes after initiation of 2 different ART strategies.

Table 2. Week 96 Characteristics of Participants Included in the Substudy

Median and IQR	RAL+DRVV/r (n = 104)	TDF/FTC+DRV/r (n = 97)	*P* Value
Total participants meeting primary endpoint during follow-up, n (%)	18 (17.3)	16 (16.5)	.878
Virologic endpoint, , n (%)			
HIV RNA ≥50 copies/mL at week 32	5 (4.8)	11 (11.3)	.121
HIV RNA ≥50 copies/mL after week 32	9 (8.7)	3 (3.13)	
HIV RNA concentration <50 copies per mL, n (%)	99 (95.2)	89 (91.8)	.322
Time to HIV RNA <50 copies per mL, median weeks (IQR)	8 (4–12.6)	18 (9.4–24.1)	<.001
CD4 cell count, cells/mm^3	597.06 (202.15)	568.39 (206.38)	.323
CD4 cell count change, cells/mm^3	265.52 (159.64)	253.40 (167.43)	.602
CD8 cell count, cells /mm^3	811.36 (329.03)	811.54 (410.93)	.997
CD8 cell count change, cells /mm^3	−123.90 (442.25)	−124.89 (350.35)	.987
CD4/CD8 ratio	0.83 (0.36)	0.82 (0.40)	.333
CD4/CD8 ratio change	0.35 (0.82)	0.43 (0.29)	.855
% with CD4/CD8 >0.4, n (%)	94 (90.4)	82 (85.4)	.269
Time to CD4/CD8 >0.4, median weeks (IQR)	24 (0.1–25)	24 (0.1–25)	.551
% with CD4/CD8 >1, n (%)	35 (33.7)	29 (29.9)	.604

Abbreviations: DRV/r, darunavir/ ritonavir; FTC, emtricitabine; IQR, interquartile range; IVDU, intravenous drug use; RAL, raltegravir; TDF, tenofovir disoproxil fumarate.

Two small prior studies have reported that participants starting ART experienced increases in mean TL [8, 17] driven by a uniform increase in the TL of CD8 T cells that correlated with a decrease in mature memory cells. Changes in the TL of CD4 T cells were more inconsistent and variable. Given these results, our hypothesis for the observed differences in TL between the two strategies in our study is that participants receiving tenofovir disoproxil fumarate/emtricitabine experienced larger increases in the TL of mainly CD8 cells and that this increase represents a shift towards less-mature T8 cell phenotypes with longer TL. Support for this hypothesis comes from the fact that 6 months after starting ART there is a decrease in proportion of $CD28^{-}CD8^{+}$ that characteristically have shorter TL and an increase in central memory T cells that have longer TL [9]. Interestingly the other predictive factor of lower TL gain in our study, alcohol consumption, increases

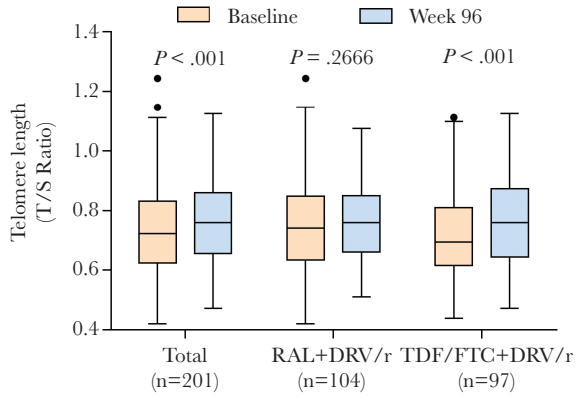

Figure 1. Blood telomere length in naive HIV-participants starting antiretroviral treatment. Abbreviations: DRV/r, darunavir/ ritonavir; FTC, emtricitabine; RAL, raltegravir; TDF, tenofovir disoproxil fumarate; T/S, telomere to single-copy gene ratio.

$CD8^{+}$ T-cell immunosenescence in simian immunodeficiency virus-infected rhesus macaques [18].

After starting ART, the main driver for immune reconstitution and a shift towards T-cell subpopulations with longer TL (naive and central memory) is the decrease of antigenic stimulation secondary to rapid control of HIV replication. In the parent NEAT 001/ANRS 143 trial although the N(t)RTI sparing regimen met noninferiority criteria for the primary endpoint, there were important differences in efficacy in favor of the tenofovir disoproxil fumarate/emtricitabine arm in the subgroup of participants with viral loads above 100 000 HIV RNA copies/mL and/or CD4 cell counts under 200 cells/μL [13]. It is therefore possible that differences in TL between groups in our substudy could be due to worse virological control with the N(t) RTI-sparing strategy. In our random sample of participants differences in blood TL occurred despite both groups having similar control of plasma viral replication and similar number of primary and virological endpoints. Notwithstanding, at week 96 plasma virological suppression was numerically higher, and occurred sooner, in participants receiving raltegravir than in those receiving tenofovir disoproxil fumarate/emtricitabine. A possible explanation is that plasma viral load may not completely reflect the antiviral efficacy of ART in tissues, especially in lymph nodes. Three recent studies have reported that compared to tenofovir disoproxil fumarate and emtricitabine, both darunavir and raltegravir have lower concentrations in lymph node tissue [19–21]. We hypothesize that participants receiving darunavir and raltegravir could have persistent HIV replication in lymph nodes (due to lower tissue concentrations) than participants receiving darunavir and tenofovir disoproxil fumarate/emtricitabine. This persistent antigenic stimulation in lymph nodes would maintain the stimulus for T cells to differentiate to

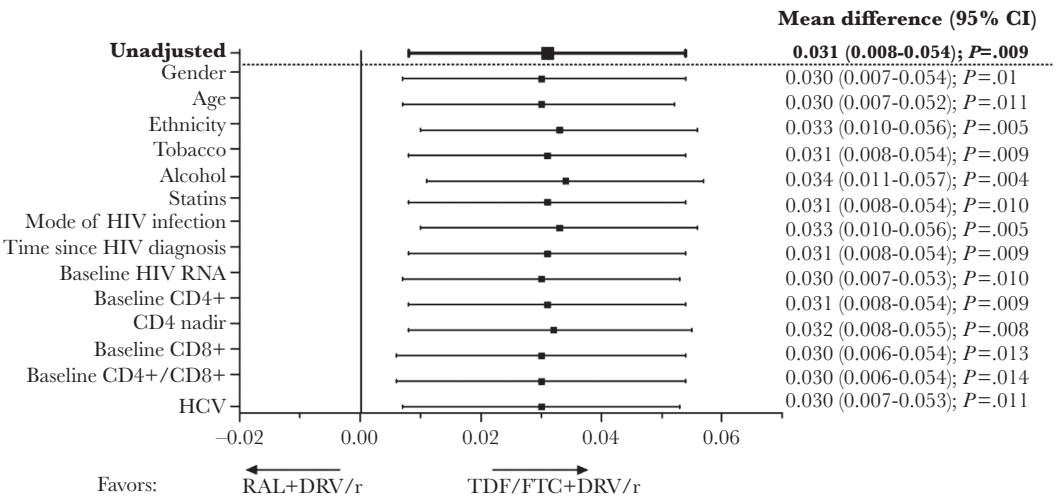

Figure 2. Mean differences between groups in telomere length (TL) change at week 96. Unadjusted and adjusted by confounders (all adjusted by baseline TL). TL measured as ratio of telomere to single-copy gene (T/S). Mean differences (95% confidence interval [CI]) from estimative multivariable regression models with TL change at week 96 as the dependent variable, treatment group as main exposure variable and each potential confounder: gender (male, female), age, ethnicity (Asian, Black, Caucasian, other) alcohol (non/moderate , exdrinker, current drinker, unknown) and tobacco (never, stopped, currently) consumption, HCV coinfection (negative, nonactive, active, unknown), statin treatment (yes, no), HIV mechanism of acquisition (homo/bisexual, heterosexual, others, unknown), time since HIV diagnosis, HIV CDC clinical stage (A, B, C), CD4 cell count, nadir CD4 cell count, CD8 cell count, CD4/CD8 ratio, log10 HIV-1 RNA, HIV-1 RNA (<100 000 c/mL, ≥100 000 c/mL). All the models were also adjusted by baseline TL. Abbreviations: DRV/r, darunavir/ ritonavir; FTC, emtricitabine; RAL, raltegravir; TDF, tenofovir disoproxil fumarate.

Table 3. Predictive Univariate and Multivariable Models for Determinants of Telomere Length Change

	Univariate		Multivariable	
	Mean Difference[a] (95% CI)	*P*	Mean Difference (95% CI)	*P*
TDF/FTC	0.031 (0.008–0.054)	.009	0.033 (0.010–0.056)	.005
Gender (ref. male)	0.016 (–0.022 to 0.053)	.410		
Younger age (per 10 years)	0.013 (0.025–0.000)	.042	0.001 (0.000–0.002)	.097
Ethnicity (ref. Asian)				
Black	0.048 (–0.053 to 0.149)	.351		
Caucasian	0.022 (–0.074 to 0.118)	.651		
Other	–0.022 (–0.136 to 0.092)	.707		
Tobacco (ref. never)				
Stopped	–0.024 (–0.066 to 0.018)	.263		
Currently	0.007 (–0.018 to 0.032)	.594		
Alcohol (ref. non/moderate)				
Exdrinker	0.075 (–0.041 to 0.191)	.202	0.073 (–0.040 to 0.186)	.205
Current drinker	–0.048 (–0.094 to –0.003)	.038	–0.052 (–0.097 to –0.006)	.026
Statin treatment (ref. No)	–0.007 (–0.051 to 0.037)	.755		
HIV mechanism of acquisition (homo/bisexual)				
Heterosexual	0.015 (–0.013 to 0.043)	.287		
Other	–0.003 (–0.121 to 0.115)	.965		
Time since HIV diagnosis (years)	0.001 (–0.002 to 0.005)	.481		
HIV-1 RNA ≥ 100 000 copies/mL	–0.012 (–0.038 to 0.013)	.339		
Nadir CD4 cell count (per 100 cells)	0.003 (–0.007 to 0.013)	.612		
Baseline CD4 cell count (per 100 cells)	0.002 (–0.007 to 0.011)	.637		
Baseline CD8 cell count (per 100 cells)	0.001 (–0.002 to 0.003)	.566		
Baseline CD4/CD8 ratio	0.001 (–0.020 to 0.021)	.956		
HCV coinfection (ref. negative)				
Active	–0.016 (–0.112 to 0.081)	.751		
Nonactive	0.103 (–0.063 to 0.268)	.222		
Baseline TL	–0.290 (–0.367 to –0.214)	<.001	–0.324 (–0.408 to –0.241)	<.001

Abbreviations: FTC, emtricitabine; TDF, tenofovir disoproxil fumarate; TL, telomere length.

[a]Adjusted by baseline telomere length.

mature phenotypes with shorter TL. In naive participants with high levels of HIV replication a higher penetration of tenofovir disoproxil fumarate/emtricitabine in lymph node tissue may overcome the inhibitory effect of tenofovir upon telomerase [10–12]. We have shown that, in the setting of virological suppression, tenofovir has a negative impact on longitudinal TL changes, [22] further supporting that in naive participants the main driver of immunosenescence is active HIV replication.

An alternative explanation for our findings is that differences in blood TL indicate an increase in T cells with shorter TL in participants treated with darunavir and raltegravir due to better control of virological replication and earlier decrease of immune activation. Individuals chronically infected with HIV have low proportions of $CD28^{-}CD8^{+}$ T cells expressing CD57, which are characterized by very short telomeres [23]. After ART initiation, the proportion of $CD28^{-}CD8^{+}$ T cells completing terminal differentiation and expressing CD57 increases [24]. Therefore, is possible that participants treated with darunavir and raltegravir do not experience blood TL increase due to lower immune activation and increasing numbers of $CD28^{-}CD57^{+}CD8^{+}$ T cells. We consider this possibility less likely: firstly because overall results of NEAT 001 indicate lower efficacy of the darunavir and raltegravir regimen, and secondly because the net effect of successful ART is to increase TL (as has been seen in several studies [8, 17], including our prospective cohort of virologically suppressed participants) [22].

Significant differences in blood TL occurred despite similar changes in CD4 and CD8 cell counts and similar CD4/CD8 ratios. In virologically suppressed participants with CD4 cell counts above 500 cells/μL, the CD4/CD8 ratio is correlated positively with the frequency of T cells with longer telomeres (naive T cells, central memory CD8, and transitional memory CD8) and negatively with the frequency of T cells with shorter telomeres (effector memory and terminally differentiated cells) [25]. However, in our study, despite the large difference observed in TL by treatment arm, there were no differences in CD4/CD8 ratio or in time to achieve a CD4/CD8 ratio above 0.4, a cutoff that in one study identified individuals with prominent immunosenescence [25]. Our data suggest that the CD4/CD8 ratio may not be sensitive enough to identify differences in the distribution of T-cell subpopulations with different TL within the first 2 years of initial ART. In our study we unveil important TL differences between 2 different ART strategies despite similar control of viral replication in blood and similar changes in CD4, CD8, and CD4/CD8 ratios. Given these results, the use of blood TL to evaluate the immunological impact of initial ART could become an interesting subject of research.

The main limitation of our study is that we did not determine TL on specific subsets of T cells. Consequently, we cannot prove at this time our hypothesis that blood TL changes are driven by modifications in T-cell subpopulations. The other limitation is the lack of samples beyond week 96. Without these long-term samples it is not possible to evaluate the long-term evolution of the observed differences between study arms.

In summary, in antiretroviral-naive participants, N(t)RTI-sparing ART using ritonavir-boosted darunavir and raltegravir was associated with lower longitudinal gains in blood TL than N(t)RTI containing ART using ritonavir-boosted darunavir, emtricitabine and tenofovir disoproxil fumarate. These results suggest that N(t)RTI-containing ART produces a more rapid initial recovery from HIV-associated immunosenescence.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We are grateful to all study participants.

Financial support. This work was supported by NEAT-ID Foundation (not for profit private foundation to promote research and education projects in the HIV field); Red Temática Cooperativa de Investigación en Sida; and Fondo de Investigaciones Sanitarias (supported by FEDER funds; grant number PI13/01467) predoctoral fellowship to N. S.-A. and RíoHortega fellowship to R.d.M. The NEAT 001/ANRS 143 trial was supported by Gilead Sciences, Janssen Pharmaceuticals, and Merck Laboratories. French National Institute for Health and Medical Research–France Recherche Nord and Sud Sida-HIV Hepatites (Inserm-ANRS) was the sponsor and a funder of the trial.

Potential conflicts of interest. N. S.-A. reports personal fees from Janssen and personal fees from Gilead outside the submitted work. R. M. received payment for development of educational lectures from Janssen. C. S. reports grants from European Commission, grants and nonfinancial support from Gilead, Janssen Cilag Pharmaceuticals, and Merck Sharp and Dohme, and grants from ANRS during the conduct of the study. J. I. B. received payment for lectures including service on the speaker's bureau from ViiV Healthcare and Janssen Cilag and currently received payment for expert testimony from Gilead Sciences and Merck Sharp and Dohme. C. A. reports personal fees and non-financial support from Janssen, Merck Sharp and Dohme, and Gilead, outside the submitted work. M. G. reports grants from Gilead, and personal fees from Merck Sharp and Dohme, Gilead, Janssen Cilag, GSK/ViiV Healthcare, and Bristol Myers Squibb outside the submitted work. C. W. reports grants from European Commission, and grants and non-financial support from Gilead, Janssen Cilag Pharmaceuticals, Merck Sharp and Dohme, and ANRS, during the conduct of the study. F. R. reports personal fees and non-financial support from Gilead Sciences, Janssen Cilag, Merck Sharp and Dohme, Merck Sharp and Dohme, and ViiV Healthcare, outside the submitted work. J. R. A. is currently receiving payment for board membership, consultancy, and lectures from ViiV Healthcare, Janssen Cilag, Gilead, and Merck Sharp and Dohme. All other authors

report no potential conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Material suplementario

Supplementary Table S1**.** Blood telomere length (TL) changes in first 101 samples

		TOTAL	RAL+DRV/r N=52	TDF/FTC + DRV/r N=49	p-value
Baseline	Mean TL (SD)	0.758 (0.151)	0.761 (0.148)	0.754 (0.156)	0.837
Week 96	Mean TL (SD)	0.786 (0.133)	0.768 (0.133)	0.805 (0.131)	
Mean TL change (95% CI)		0.028 (0.009; 0.005)	0.007 (-0.015; 0.030)	0.050 (0.018; 0.083)	0.016*
N (%) with TL gain at W96		65 (64.36)	29 (55.77)	36 (73.47)	0.067
N (%) with TL gain >1 SD at W96		23 (22.77)	7 (13.46)	16 (32.65)	0.022

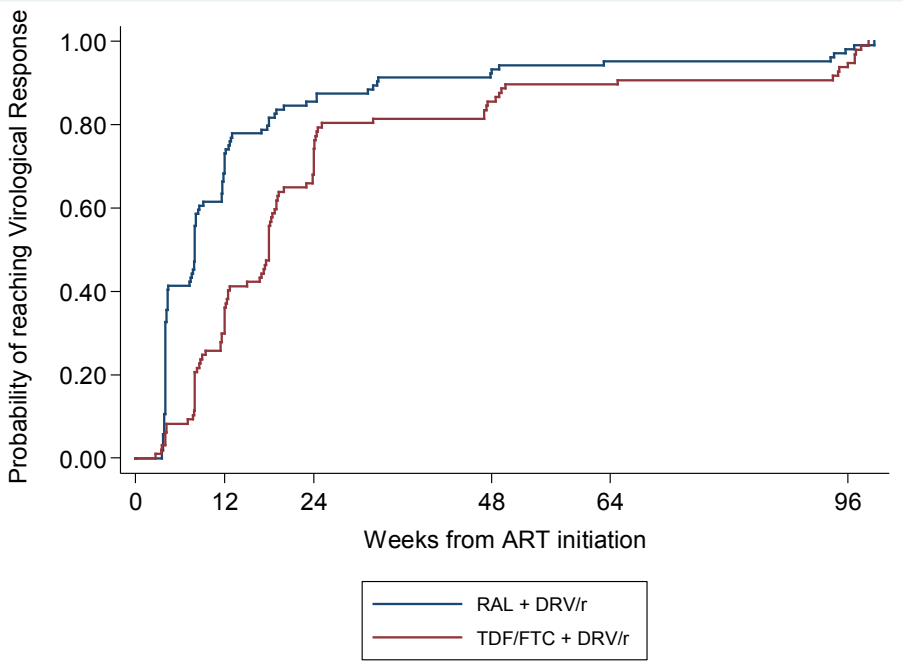
*Adjusted by baseline telomere

Table S2. Blood telomere length (TL) changes

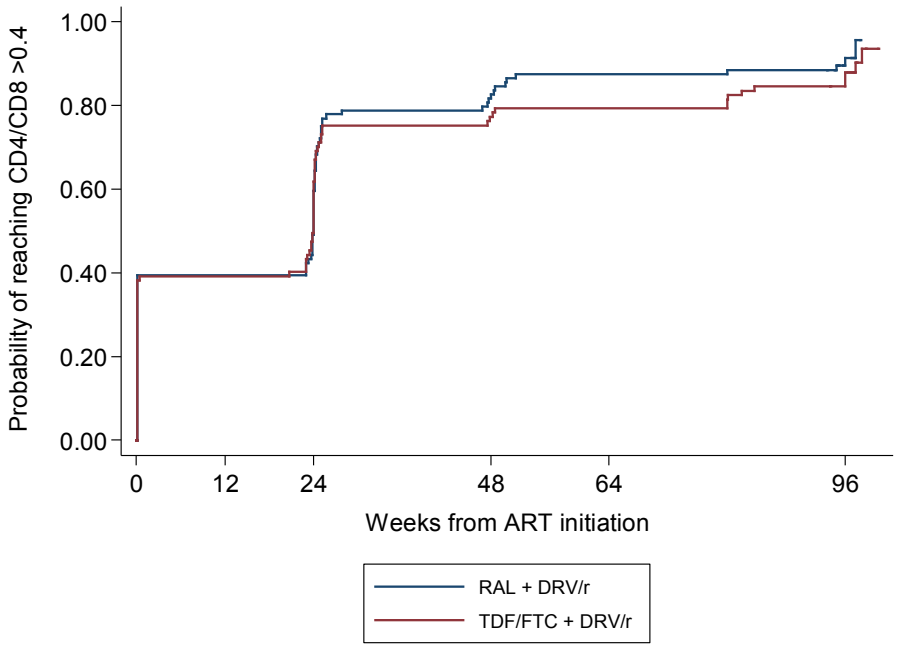
		TOTAL	RAL+DRV/r N=104	TDF/FTC + DRV/r N=97	p-value
Baseline	Mean TL (SD)	0.738 (0.152)	0.750 (0.154)	0.724 (0.149)	0.221
Week 96	Mean TL (SD)	0.766 (0.136)	0.760 (0.133)	0.772 (0.140)	
Mean TL change (95% CI)		0.028 (0.015,0.041)	0.009 (-0.007, 0.026)	0.048 (0.028, 0.067)	0.009*
N (%) with TL gain at W96		128 (63.68)	59 (56.73)	69 (71.13)	0.034
N (%) with TL gain >1 SD at W96		47 (23.38)	21 (20.19)	26 (26.80)	0.268

*Adjusted by baseline telomere

Supplementary Figure S1. Time to Virological Response (RNA Viral Load <50 copies/ml)



Supplementary Figure S2. Time from ART initiation to CD4/CD8 ratio > 0.4



Discusión

Gracias al indiscutible éxito del TAR la esperanza de vida del paciente VIH ha aumentado significativamente hasta aproximarse a la de la persona no infectada. Sin embargo, el TAR no consigue restaurar por completo la salud del paciente por lo que la población VIH+ presenta múltiples comorbilidades asociadas al envejecimiento a edades menos avanzadas que en la población no VIH. Dado que en población general existe una asociación entre las enfermedades asociadas al envejecimiento y el acortamiento telomérico, y además existe una similitud estructural y funcional entre la transcriptasa inversa del VIH y la telomerasa celular, el trabajo realizado en esta tesis doctoral se ha centrado en el estudio del impacto de los N(t)RTIs en la actividad telomerasa *in vitro* y su impacto en la LT *in vivo* tanto en pacientes con prolongada supresión virológica como pacientes naïve que comienzan TAR de primera línea.

En el estudio *in vitro* hemos confirmado que concentraciones terapéuticas de TFV y ABC producen una disminución de la actividad telomerasa dosis-dependiente, siendo TFV el inhibidor más potente. Tras el tratamiento de PBMCs con cada uno de los fármacos, se obtuvo 29% de inhibición con TFV y 12% de inhibición con ABC. Al contrario de lo observado en el trabajo de Leeansyah *et al* [81], con FTC no se detectó disminución de actividad telomerasa, ni siquiera a altas concentraciones del fármaco. Además se evaluó si el tratamiento con DRV podría aumentar la actividad telomerasa, tal y como se había observado en estudios previos con el inhibidor de proteasa Saquinavir [83–85], sin embargo los resultados obtenidos indican que la actividad telomerasa no se ve alterada en presencia de DRV. Finalmente, se determinó que la disminución de la actividad telomerasa observada con TFV y ABC no se asociaba a cambios en los niveles de la subunidad catalítica TERT, o en la expresión de los genes que codifican para TERT u otras subunidades de la telomerasa o del complejo shelterina.

Las formas fosforiladas de TFV, ABC y FTC compiten con los deoxinucleótidos trifosfato intracelulares de A, G y C respectivamente e inhiben la transcripción reversa viral debido a que son terminadores de cadena. Por lo tanto, la inhibición de la actividad telomerasa por los N(t)RTIs solo es posible con TFV y ABC pero no con FTC. Leeansyah *et al* han demostrado previamente que TDF a concentraciones terapéuticas es un potente inhibidor de la telomerasa *in vitro* y que dicha inhibición se asocia al acortamiento telomérico [81]. Además, detectaron inhibición de la actividad telomerasa con ABC y FTC a concentraciones superiores a sus rangos terapéuticos, sin embargo dicha inhibición no se asoció a acortamiento telomérico.

El siguiente objetivo de esta tesis consistió en estudiar si la inhibición de la actividad telomerasa *in vitro* causada por los N(t)RTIs, y en especial TDF, tiene alguna relevancia *in vivo*. Para ello hemos analizado muestras de dos poblaciones diferentes de pacientes VIH.+: 1) una cohorte de pacientes con prolongada supresión virológica del Hospital Universitario La Paz, que fueron seleccionados en función de la exposición o no a TDF y 2) una muestra de pacientes naïve del ensayo clínico NEAT 001/ANRS 143 que habían sido randomizados a empezar TAR con una pauta que incluía 2 N(t)RTIs (DRV/r + TDF/FTC) o con una pauta ahorradora de N(t)RTIs (DRV/r + RAL).

El estudio *in vivo* realizado en la cohorte de pacientes con supresión virológica prolongada ha consistido en un primer estudio de corte transversal y, tras dos años de seguimiento de los pacientes de la cohorte, un estudio longitudinal. Los resultados obtenidos en el estudio transversal mostraron que la historia de exposición a TDF no tiene un impacto negativo en la LT medida en sangre completa. El análisis multivariante mostró que en los pacientes expuestos a TDF por períodos prolongados, 70% de ellos por más de 5 años, la LT en sangre no difería significativamente de la de los pacientes que nunca estuvieron expuestos. Por el contrario, en el estudio longitudinal sí se encontraron diferencias en el incremento de LT entre los grupos. De forma global se observó una ganancia de la LT en sangre tras dos años de seguimiento. Al analizar la LT en función de la exposición a TDF se observó que, aquellos pacientes continuamente expuestos a TDF presentaban una ganancia de la LT significativamente inferior a la de los pacientes nunca expuestos a TDF. Esto mismo se observó al analizar la LT en función de la estrategia de TAR, aquellos pacientes cuyo TAR incluía TDF o ABC presentaban una ganancia de la LT significativamente inferior a la de los pacientes cuyo TAR no incluía N(t)RTIs. Sin embargo, no hubo diferencias significativas en ganancia de LT entre los pacientes cuyo TAR incluía TDF o ABC.

Es importante destacar que existen diferencias en el criterio de inclusión de los pacientes expuestos a TDF entre el estudio transversal y el longitudinal. En el estudio de corte transversal los pacientes incluidos en el grupo con exposición a TDF habían recibido en algún momento de su TAR una pauta que incluía TDF, sin embargo al momento del inicio del estudio sólo el 55% de los pacientes estaban recibiendo TDF. Por el contrario, en el estudio longitudinal se incluyeron en el grupo con exposición a TDF sólo a aquellos pacientes que estaban recibiendo TDF como parte del TAR al momento del inicio del estudio. Por lo tanto, teniendo en cuenta esta diferencia en el criterio de selección de los pacientes expuestos a TDF, los resultados sugieren que el impacto negativo de TDF en los cambios de la LT en sangre podría ser reversible tras interrumpirse el tratamiento con TDF.

Tanto en el estudio transversal como en el longitudinal, las características basales de los pacientes expuestos a TDF y nunca expuestos a TDF eran comparables en relación a aquellos factores que se han asociado previamente a la LT tales como sexo, edad, ingresos y hábito tabáquico. Además, dado que el propio VIH inhibe la actividad telomerasa [72,86], para minimizar este efecto un criterio de selección fue que los pacientes tuviesen la carga viral suprimida durante al menos un año. Por lo tanto, no hubo diferencias en el control virológico entre grupos. Finalmente, ambos grupos eran comparables en cuanto a la duración del TAR y el tiempo de exposición a N(t)RTIs, otros dos factores que podrían afectar la LT [81].

Hasta el momento, se han publicado pocos trabajos que estudian el impacto *in vivo* de los diferentes regímenes de TAR en la LT y ninguno de ellos se ha centrado particularmente en el impacto de TDF. En un estudio de corte seccional con una muestra de 50 pacientes con la carga viral ≤ 50 copias/ml, Leeansyah *et al* observaron en su análisis univariante que la duración del régimen de TAR con N(t)RTIs se asociaba inversamente con la LT, pero no con la actividad telomerasa [81]. Sin embargo, en el análisis multivariante, dicha asociación no fue significativa. Esta falta de asociación entre la LT y la duración del TAR se observa también en otros dos estudios transversales que se realizaron con un mayor tamaño muestral [13,15]. Sin embargo, es importante destacar que, en estos dos estudios transversales, no todos los pacientes estaban recibiendo TAR y solo el 59% de los pacientes en un caso y el 84% en el otro presentaban la carga viral indetectable. Finalmente, en el subestudio del ensayo clínico MONET, que evaluaba la pauta de monoterapia con DRV/r versus la triple terapia con DRV/r y 2 N(t)RTIs para el mantenimiento de la supresión virológica, se determinó que continuar con la pauta de TAR que incluía 2 N(t)RTIs no se asociaba con cambios en la LT [82]. Es importante señalar que el tamaño muestral del subestudio del ensayo MONET fue pequeño, ya que de los 124 participantes solo el 88% tenían muestra disponible tras 48 semanas. Además, se observaba una falta de balance en el momento basal del estudio ya que los pacientes asignados a interrumpir los N(t)RTIs presentaban los telómeros más largos. Por último, el análisis estadístico no ha sido ajustado por la LT basal, tal y como se recomienda para los estudios longitudinales [87], por lo tanto este subestudio del ensayo clínico MONET carece de potencia suficiente para determinar el impacto de los N(t)RTIs en los cambios de la LT.

Los resultados del estudio prospectivo concuerdan con los resultados obtenidos previamente en el estudio *in vitro*. En este estudio *in vivo* se observó un incremento de la LT en sangre independientemente de la pauta de TAR, sin embargo se apreciaban diferencias en la ganancia de LT entre diferentes pautas. La mayor diferencia en la ganancia de LT se produjo entre los pacientes cuyo TAR incluía TDF y los pacientes que recibieron una pauta ahorradora de N(t)RTIs.

Esta diferencia en la ganancia de LT resultó ser menor cuando las pautas comparadas fueron el TAR que incluía ABC y la pauta ahorradora de N(t)RTIs. Además, al comparar la ganancia de LT entre las pautas que incluían TDF y ABC, la diferencia entre ambas fue incluso menor no alcanzando la significación estadística. Por lo tanto, estos resultados apuntan a la inhibición de la telomerasa como el mecanismo subyacente responsable de las diferencias de LT observadas en esta cohorte.

El incremento de la LT en sangre parece contrario a los datos obtenidos en población general en los que se observa una disminución anual de la LT en sangre [68]. Una posible explicación sería que los pacientes VIH+ con carga viral suprimida, a pesar de tener un alto recuento de células T CD4+ y una supresión virológica de 7 años de media al inicio del estudio, aún estén experimentando reconstitución inmunológica. En una publicación reciente se ha determinado que, en aquellos pacientes que comenzaron el TAR con un recuento de CD4 ≤ 200 células/µl, la disminución del recuento de células T CD8+ y el incremento del ratio CD4/CD8 se prolonga hasta 15 años desde el inicio del TAR [88]. Además, en el estudio de Behrens *et al* los pacientes con prolongada supresión virológica muestran una reducción significativa de las células T terminalmente diferenciadas, sugiriendo una disminución de la senescencia, y una mejora de la proporción de células naïve/memoria [89]. Por lo tanto, el aumento de la LT observada sería consecuencia de un cambio de las subpoblaciones de linfocitos T hacia fenotipos menos maduros que presentan telómeros más largos. Debido a que la ganancia de la LT fue menor en los pacientes cuyas pautas de TAR incluían TDF o ABC y dado que el acortamiento telomérico es una de las características de la senescencia de células T, estos resultados sugieren que TDF y ABC podrían interferir en el proceso de recuperación de la inmunosenescencia.

Se desconoce la relevancia clínica de las diferencias de la LT en sangre encontradas en esta cohorte. Un estudio reciente en 51 usuarios de drogas inyectables determinó que 3 meses después de la seroconversión la LT en PBMCs, medida por qPCR, disminuyó un 13% [67]. En el análisis por protocolo de este estudio longitudinal *in vivo*, la diferencia ajustada de la ganancia media de LT en sangre a semana 96, entre los pacientes expuestos a TDF y nunca expuestos, fue del 3% (Figura 8). Además, la diferencia de LT entre las distintas pautas del TAR se incrementa tras dos años de seguimiento, lo que sugiere un impacto acumulativo de los N(t)RTIs en la LT. Por lo tanto, el principal factor asociado al acortamiento telomérico es la propia infección por el VIH, sin embargo cuando la replicación viral está suprimida el impacto de la inhibición de la telomerasa por los N(t)RTIs, y por tanto la menor ganancia de la LT observada en el grupo con exposición a TDF, adquiere relevancia.

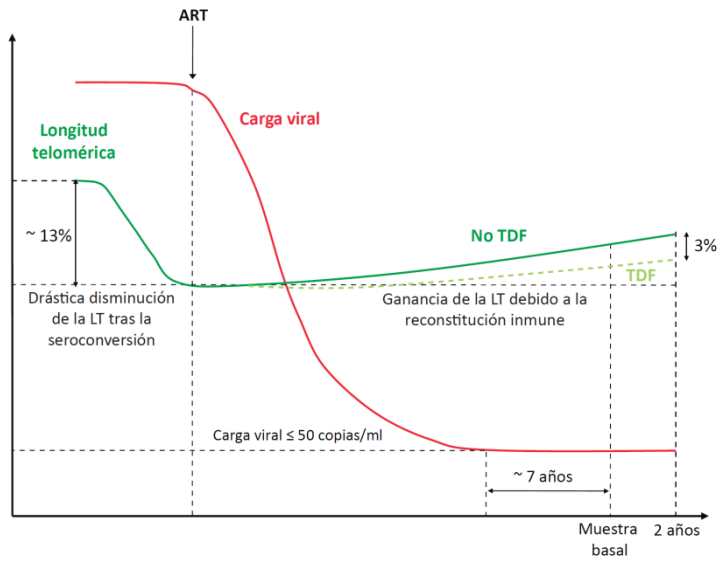


Figura 8 | Esquema de la ganancia de LT en la cohorte de pacientes con supresión viral prolongada tras dos años de seguimiento. Se observa una diferencia en la ganancia de la LT entre los pacientes cuyo TAR incluye TDF y aquellos nunca expuestos a TDF, siendo la ganancia de LT un 3% menor en el grupo de pacientes expuestos a TDF.

Al no tratatse de un estudio aleatorizado, nuestra cohorte prospectiva no permite determinar si variables que no han sido medidas podrían influir en el cambio de la LT en sangre. Para evitar los sesgos inherentes a los estudios de cohortes realizamos un nuevo estudio longitudinal anidado en un ensayo clínico. Determinamos LT en sangre de una muestra aleatoria de 201 pacientes del ensayo clínico NEAT 001/ANRS 143. En dicho ensayo clínico, pacientes naïve VIH+ fueron aleatorizados en dos grupos: empezar TAR con una pauta que incluía 2 N(t)RTIs (DRV/r + TDF/FTC) o con una pauta ahorradora de N(t)RTIs (DRV/r + RAL) [90]. Contrariamente a lo esperado en función de los resultados obtenidos previamente en los estudios *in vitro* e *in vivo*, los pacientes que recibieron la pauta DRV/r + TDF/FTC tuvieron una significativa mayor ganancia en la LT en sangre que los pacientes que recibieron la pauta DRV/r + RAL. Estos resultados sugieren que incluir N(t)RTIs en la pauta de TAR podría tener un papel importante en la recuperación inicial de la inmunosenescencia asociada al VIH.

Al igual que lo observado en el estudio longitudinal de la cohorte, la media de la LT en sangre aumentó de forma global tras dos años de TAR. Sin embargo, el incremento sólo fue significativo en los pacientes que recibieron la pauta DRV/r + TDF/FTC siendo la proporción de pacientes con incremento de la LT un 14% mayor que en el grupo con la pauta ahorradora de N(t)RTIs. En el análisis estimativo, la diferencia ajustada de la ganancia media de LT entre los grupos fue del 4.2% (Figura 9). Dado que se ha determinado en PBMCs que el acortamiento del telómero tras la seroconversión es del 13% [67], cabe considerar que las diferencias encontradas en la recuperación de la LT sean relevantes.

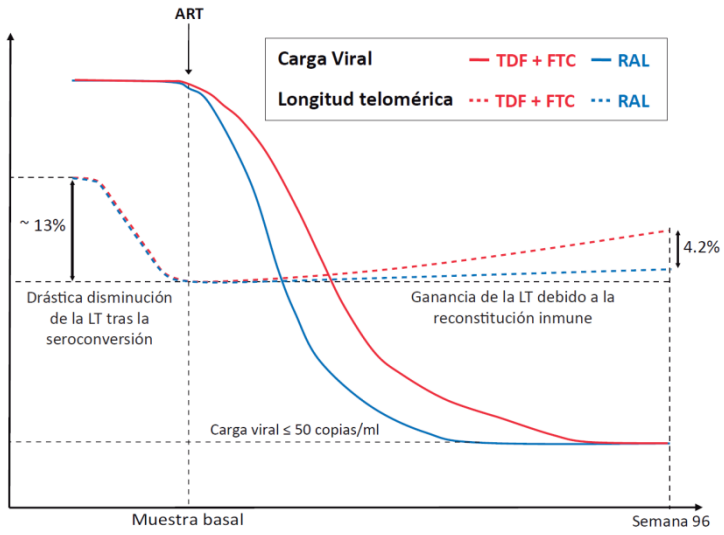


Figura 9 | Esquema de la ganancia de LT tras el inicio del TAR de primera línea. Se observa una diferencia en la ganancia de la LT a semana 96 entre los pacientes cuyo TAR incluye TDF/FTC y aquellos que reciben RAL, siendo la ganancia de LT un 4,2% mayor en la rama que incluye TDF/FTC.

No se detectaron variables basales o de semana 96 que pudiesen sesgar o alterar el efecto de TDF/FTC en la ganancia telomérica. En el análisis predictivo se observa que el factor asociado a una mayor ganancia de la LT fue el TAR con TDF/FTC y que el factor asociado a una menor ganancia de la LT fue el consumo de alcohol. Se ha determinado previamente que el abuso de alcohol se asocia al acortamiento telomérico en población no VIH [91]. En el caso de la edad no se alcanza la significación estadística, aunque se observa una tendencia hacia una mayor ganancia de LT en los individuos de menor edad.

El NEAT 001/ANRS 143 ha sido el primer ensayo clínico en el que se han comparado cambios en la LT en sangre tras el inicio de dos pautas diferentes de TAR. Dos pequeños estudios previos habían observado que los pacientes que inician TAR experimentan un incremento en la LT media [75,92]. Dicho incremento se asocia al aumento de LT que se produce en células T CD8+ y además se correlaciona con una disminución de la población de células de memoria. Por el contrario, los cambios de la LT de las células T CD4+ tras el inicio del TAR fueron más variables e inconsistentes. En función de estos resultados, se podría considerar que las diferencias observadas en la LT entre las dos pautas de TAR se deben a que los pacientes que recibieron TDF/FTC experimentaron un mayor incremento en la LT principalmente en células T CD8+ y que dicho incremento representa un cambio de la población CD8+ hacia células con fenotipos menos maduros que presentan telómeros más largos. Además, en un trabajo reciente se ha observado que 6 meses después de comenzar el TAR de primera línea se detecta una disminución de la proporción de la subpoblación de células T CD8+ CD28-, que se caracterizan por tener telómeros

cortos, y un incremento en las células de memoria central CD8+ CD28+ cuya LT es mayor [93]. Cabe destacar que el consumo de alcohol, el otro factor predictivo de menor ganancia de LT en este subestudio, se ha visto que aumenta la inmunosenescencia de células T CD8+ en macacos Rhesus infectados con el virus de la inmunodeficiencia del simio [94].

Después del comienzo del TAR, la principal causa de la reconstitución inmune y el cambio hacia subpoblaciones de células T con mayor LT, tales como célula naïve y de memoria central, es la disminución del estímulo antigénico debido al control de la replicación viral. En el estudio parental NEAT 001/ARNS 143 la pauta ahorradora de N(t)RTIs alcanzó la no inferioridad, aunque se observaron importantes diferencias en favor de TDF/FTC en el subgrupo de pacientes con recuento de células CD4+ inferior a 200 células/µl o carga viral igual o superior a 100.000 copias/ml [90]. Por lo tanto, en este subestudio del NEAT cabría pensar que la diferencia detectada en la ganancia de la LT entre ambos grupos se debe al peor control virológico de la pauta ahorradora de N(t)RTIs sin embargo, no se observaron diferencias en el control de la carga viral o en el número de endpoints primarios y virológicos en el análisis restringido a los participantes incluídos en el subestudio. Nuestra hipótesis para reconciliar estos resultados es que la carga viral en plasma podría no reflejar completamente la eficacia antirretroviral del TAR en los tejidos, especialmente en los ganglios linfáticos. Estudios recientes han observado que comparado con TDF/FTC tanto DRV como RAL presentan menores concentraciones en ganglios linfáticos [95–97]. Debido a esta diferencia en la concentración de los fármacos, se podría pensar que el grupo de pacientes con la pauta DRV + RAL presenta una replicación viral persistente en ganglios linfáticos. Dicho estímulo antigénico persistente en los ganglios linfáticos podría ser responsable de la diferenciación de las células T hacia fenotipos más maduros con telómeros más cortos. De esta manera, en el caso de los pacientes naïve con una mayor replicación viral en plasma, una mejor penetración de TDF/FTC en ganglios linfáticos podría ser más relevante que el efecto inhibitorio de TDF sobre la actividad telomerasa [76,81].

Un estudio realizado en pacientes VIH+ con la carga viral suprimida y un recuento de CD4+ > 500 células/µl ha observado que el bajo ratio CD4/CD8 se asocia con una disminución de la frecuencia de células T CD8+ naïve y de memoria central y con un incremento de la frecuencia de las células T CD8+ terminalmente diferenciadas, como así también con altos niveles de células T CD8+ activadas y senescentes [98]. Además, en este estudio los pacientes cuya ratio CD4/CD8 fue inferior a 0,4 se caracterizaron por presentar una avanzada inmunosenescencia. En el subestudio del NEAT ambos grupos alcanzaron un recuento de células CD4+ superior a 500 células/µl a semana 96. Sin embargo, a pesar de la diferencia de la ganancia de LT observada entre ambas pautas de TAR, no se observaron diferencias en el ratio CD4/CD8, o el tiempo hasta

alcanzar un ratio CD4/CD8 superior a 0,4. Estos resultados sugieren que, tras dos años desde el inicio del TAR, el ratio CD4/CD8 podría no ser suficientemente sensible para identificar diferencias en la distribución de las subpoblaciones de células T con diferente LT. Dado que además, en este subestudio no se observaron diferencias en relación al control de la replicación viral o el cambio de las proporciones de células CD4+, CD8+ o del ratio CD4/CD8, el uso de la LT en sangre para evaluar el impacto inmunológico del TAR inicial podría ser un interesante tema para investigaciones futuras.

Como se ha explicado a lo largo de la discusión, nuestra hipótesis es que la ganancia de la LT sería consecuencia de la reconstitución inmune y presumiblemente se asocia a un cambio en las subpoblaciones de linfocitos T hacia fenotipos menos maduros que presentan telómeros más largos. Sin embargo, en el desarrollo de esta tesis doctoral no hemos determinado la LT en las poblaciones de células T CD4+ y CD8+, ni hemos estimado la proporción de las subpoblaciones linfocitarias de células naïve, de memoria o senescentes. Para investigar esta hipótesis ya hemos iniciado dos nuevos estudios con el fin de evaluar la LT en dichas poblaciones celulares. El primer estudio corresponde al seguimiento a cuatro años de los pacientes con supresión virológica de la cohorte del Hospital Universitario La Paz, en el cual se evaluará la LT no solo en sangre total sino también en las poblaciones de células T CD4+ y CD8+ como así también la actividad telomerasa en células T CD4+ y CD8+. Además, mediante el uso de citometría, se determinará la proporción de las subpoblaciones linfocitarias de células naïve, de memoria, activadas, senescentes y exhaustas tanto CD4+ como CD8+. Finalmente, este estudio también permitirá determinar si la diferencia en la ganancia de LT en sangre observada a los dos años de seguimiento continúa incrementándose, o si por el contrario se mantiene estable o incluso disminuye. En el segundo estudio se estimará la abundancia de las células T CD4+ y CD8+ como así también las subpoblaciones de células naïve y senescentes en la población de pacientes naïve del ensayo clínico NEAT001/ANRS143. Dado que, en este estudio sólo disponemos de muestras de sangre completa, para realizar dicha determinación se utilizará el abordaje de análisis epigenético mediante patrones de metilación del ADN descrito previamente por Chen *et al* [99]. Este abordaje permitirá estudiar el cambio de la abundancia de la células T CD4+ y CD8+ tras el inicio del TAR y evaluar su correlación con el cambio de la LT determinado previamente en sangre.

En resumen, en esta tesis doctoral que se caracteriza por su marcado enfoque traslacional, los resultados obtenidos *in vitro* proporcionan más evidencia sobre la inhibición de la actividad telomerasa causada por algunos N(t)RTIs, concretamente TDF y ABC, siendo TDF el fármaco que produce la mayor inhibición a concentraciones terapéuticas. El principal factor asociado al

acortamiento telomérico es la propia infección por el VIH, sin embargo cuando la replicación viral está suprimida la inhibición de la telomerasa causada por los TDF o ABC parece ser relevante. Tanto en el estudio *in vivo* realizado en la cohorte de pacientes con prolongada supresión virológica como en el subestudio del NEAT con pacientes naïve que comienzan TAR de primera línea, se ha observado que el TAR tuvo un impacto positivo en los cambios de LT observados tras dos años de seguimiento independientemente de la pauta de tratamiento. Dicha ganancia de la LT presumiblemente sea el resultado de cambios en las subpoblaciones de células T hacia fenotipos menos maduros que presentan telómeros más largos. Sin embargo, al analizar la ganancia de la LT en función de la pauta de TAR se observaron diferencias entre ambos estudios. En el estudio de la cohorte de pacientes avirémicos se ha observado que las pautas de TAR que incluían TDF o ABC se asociaron a una menor ganancia de la LT que la pauta ahorradora de N(t)RTIs, lo que refuerza los resultados *in vitro*. Por el contrario, en el subestudio del ensayo clínico NEAT con pacientes naïve la pauta que incluía TDF/FTC se asoció a una mayor ganancia de la LT que la pauta ahorradora de N(t)RTIs que incluía RAL, lo que sugiere que los N(t)RTIs se asocian a una mejor recuperación inicial de la inmunosenescencia asociada al VIH.

Conclusiones

1. Concentraciones terapéuticas de TFV y ABC, pero no de FTC, inhiben la actividad telomerasa *in vitro*, siendo TFV el inhibidor más potente. TFV y ABC inhiben la actividad enzimática, sin afectar a los niveles de proteína ni a la expresión génica.

2. Tras dos años de seguimiento de los pacientes VIH+ con prolongada supresión viral, la LT en sangre aumentó menos en el grupo tratado con TDF o ABC que en el grupo tratado con la pauta ahorradora de N(t)RTIs, lo que sugiere que estos N(t)RTIs interfieren con la recuperación a largo plazo de la inmunosenescencia asociada a la infección por el VIH, posiblemente debido a su capacidad para inhibir la telomerasa.

3. Tras dos años de seguimiento de los pacientes naïve VIH+, la LT en sangre aumentó más en el grupo de pacientes tratados con TDF/FTC que en el grupo tratado con la pauta ahorradora de N(t)RTIs; lo que sugiere que TDF/FTC produce una mejor recuperación inicial de la inmunosenescencia asociada a la infección por el VIH.

Conclusions

1. Therapeutic concentrations of TFV and ABC, but not of FTC, inhibit telomerase activity *in vitro*, being TFV the most potent inhibitor. TFV and ABC inhibit enzymatic activity, without affecting protein levels or gene expression.

2. After two years of follow-up of HIV patients with prolonged viral suppression, blood telomere length increased less in the group of patients exposed to TDF or ABC than in the group treated with the N(t)RTIs-sparing regimen; suggesting that these N(t)RTIs interfere with the long-term recovery of immunosenescence associated with HIV infection, possibly due to their ability to inhibit telomerase.

3. After two years of follow-up of naïve HIV+ patients, blood LT increased more in the group of patients treated with TDF/FTC than in the group treated with the N(t)RTIs-sparing regimen; suggesting that TDF/FTC produces a better initial recovery of immunosenescence associated with HIV infection.

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Anexo

Publicaciones no relacionadas con la tesis doctoral

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